A polymeric chip system and uses thereof

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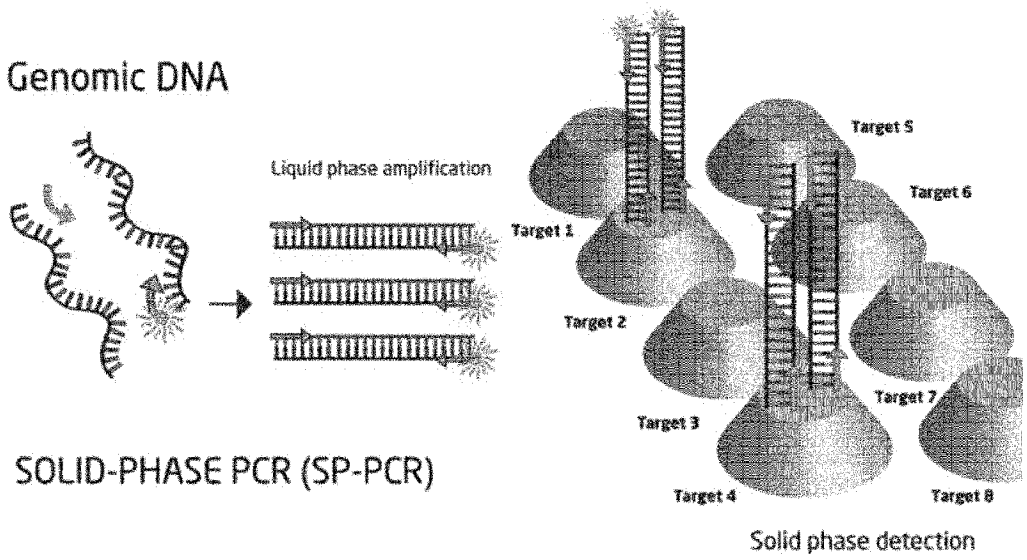

Figure 6

(57) Abstract: The invention relates to an optical chip comprising at least one, preferably a plurality of, solid optical element(s), where the optical element comprises an upper planar detection surface to which a moiety for capturing and/or detecting an analyte may be linked. The invention further provides methods of using the optical chip.

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A polymeric chip system and uses thereof

Field of the invention

The invention relates to an optical chip comprising at least one, preferably a plurality of, solid optical element(s), where the optical element comprises an upper planar detection surface to which a moiety for capturing and/or detecting an analyte may be linked. The invention further provides methods of using the optical chip.

Background of the invention

Multiplex detection methods are essential to conduct DNA-based diagnostics. In order to accurately analyze food, clinical or environmental samples, it is important to detect and differentiate not only different pathogens but also different genus, species, sub-species and serotypes of the pathogens. At the moment, the most commonly used strategy is multiplex quantitative PCR (qPCR). A qPCR assay can concurrently amplify and detect different DNA sequences in the same reaction tube through the use of specific primers, but the techniques are only able to amplify a limited number of genes due to the interferences between different primer and primer sets, as well as the limited multiplexing capability of fluorescence optical detection of the liquid phase PCR. As an alternative strategy, solid phase PCR (SP-PCR) has attracted enormous interest (Khodakov and Ellis, 2014). The technique amplifies target nucleic acids on a solid support with one or both primers attached to a surface. Since the spatial separation of primers minimizes undesirable interactions, numerous primers can be arranged in format of high-density array within a relatively small space. Therefore, SP-PCR provides much higher multiplexing capability than qPCR and holds significant promise in multi-analyte analysis (Sun et al., 2011a).

To date, molecular diagnostic industry is actively seeking simple and cost-effective sensing platforms that can be employed in point-of-use settings.

Driven by the demand, lab-on-a-chip (LOC) technology has been rapidly developed utilizing the capabilities of microfluidic technologies to integrate multiple laboratory processes in a miniaturized device. A few attempts have been done to incorporate the SP-PCR into LOC systems in order to achieve multiplexed analysis (Hoffmann et al., 2012; Sun et al., 2011b). The SP-PCRs were performed either in a microarray format within a single chamber (Sun et al., 2011b) or in multiple wells with reaction volumes down to pico litres (Hoffmann et al., 2012). However, as the amplification efficiency of the SP-PCR is relatively lower than conventional liquid PCR, a highly sensitive benchtop laser scanner is often required to detect and quantify the signals of the SP-PCR. Such scanners are bulky, expensive and take extremely long time for scanning an area with high resolution. Therefore, the techniques are not suitable for on-site testing.

Recently, a number of portable detection methods were developed for parallel readout in LOC devices. They were based on various principles, such as magnetic, electrochemical, mechanical or optical properties. Although magnetic, electrochemical and mechanical techniques possess an ability of ultrasensitive and real-time detection, their applications in SP-PCR-based microfluidic devices are greatly limited by complex fabrication procedures, strict requirements of assay optimization and precise control on differentiation between nonspecific and specific binding. In contrast, optical detection is more attractive for LOC systems as it is robust, not affected by the environmental variations, and moreover, the ability to detect multiplexed reactions in SP-PCR could simply be realized by rapidly extracting data from e.g., images of the fluorescence light. On-chip optical sensing has been achieved by either developing portable versions of conventional optical instrumentation, or by incorporating part of the optical detection system on the microfluidic substrate itself. Despite of the recent advances in compact optical sensors, inherent factors related to on-chip detection, such as the requirements of short working distances and miniaturized electronic or optical components, have led to small field of view, high optical losses and decreased signal-to-noise ratio. It remains an ongoing challenge for

integrating of optical detectors in microfluidic devices for highly sensitive and multi-point detection.

Hung et al. (2015) discloses a high-quality micro-optical lens array as part of polymeric chip with improved sensitivity of fluorescence detection. It was based on the phenomenon called super critical angle fluorescence (SAF), which reveals that for a fluorophore sitting at the surface interface, majority of the light is emitted into the material with higher refractive index, mostly around the supercritical angle. This main part of the fluorescent light, which is normally lost when using conventional microscopy, was collected using SAF micro-optical array made of polystyrene and prepared by injection moulding.

Hung et al. (2015) demonstrated a 46 fold increase in the sensitivity of the test by collecting light at the critical angle using the SAF micro-optical array. Moreover, the SAF structure offers a large field of view as the special arrangement of optical pathway makes light collection efficiency independent of the numerical aperture of the optical system. Last but not least, the SAF-micro-optical array could be fabricated in a high density, which increases the multiplexing capability for detection of biological targets.

Although Solid-phase PCR (SP-PCR) has become increasingly popular for molecular diagnosis, there have only been a few attempts to incorporate SP-PCR into lab-on-a-chip (LOC) devices. There is therefore an unmet need for simple and cost-effective lab-on-a-chip (LOC) technology that integrate the SP-PCR into LOC systems in order to achieve multiplexed analysis suitable for point-of-use applications.

US 7,750,316 B2 (MacCraith et al.) discloses an optical chip for fluorescence detection. The optical chip has one or more parabolic optical elements that capture and collimate the fluorescent light and direct it onto a detector. The optical chip may be constructed of a polymer and made using injection molding techniques.

Summary of the invention

The object of the present invention is to provide sensitive and portable on-chip optical detection technology. In particular, it is an object of the present invention is to provide a lab-on-a-chip (LOC) technology that is useful for solid phase PCR (SP-PCR) application.

A further object is to provide an improved method for conducting multiplexed analysis.

Yet a further object is to provide an improved LOC platform.

The inventors' solution to the problem is a LOC platform, which combines the SP-PCR with the SAF micro-optical array in a polymeric microfluidic chip, so that signals from highly parallel SP-PCR reactions is effectively detected on chip with low-cost and compact optical components.

A first aspect of the present invention provides an optical chip comprising at least one solid optical element, wherein said optical element

(i) has a conical frustum shape, defining a lower diameter, an upper diameter, a height, and a surface roughness (RMS roughness), and wherein said lower diameter does not exceed 350 micrometer, said upper diameter does not exceed 175 micrometer, said height does not exceed 150 micrometer, and said surface roughness (RMS roughness) does not exceed 75 nm,

(ii) comprises an upper planar detection surface defined by said upper diameter to which a detection moiety may be linked, and

wherein fluorescence emission light entering said optical element at said detection surface at an angle equal to or greater than theta(min) is totally internally reflected and (at least substantially) collimated along an axis perpendicular to said detection surface.

A second aspect of the present invention relates to a method for preparing an optical chip according to any of the preceding claims, said method comprising the steps of:

(i) providing a wafer,

(ii) coating said wafer with a photoresist which is not epoxy-based and baking said wafer with said photoresist,

(iii) providing an assembly comprising a fixed light source pointing in the direction of a rotatable holder configured to receive and hold said wafer, and wherein said rotatable holder is configured such that the angle of rotation with respect to said light source may be altered within the range of 0 to 90 degrees, such as 0 to 60 degrees,

(iii) placing said photoresist-coated wafer and a photomask in the rotatable holder such that the photomask covers and is in contact with said wafer,

(iv) exposing said photoresist-coated wafer to a light source capable of developing said photoresist, while the holder rotates with a fixed speed, preferably maximum speed of 4 seconds per rotation,

(v) removing uncross-linked photoresist outside of the SAF structures forming on the wafer of step (iv),

(vi) coating the wafer of step (v) with a layer of conducting metal,

(vii) prepare a master structure by electroplating (such as nickel-electrodeposition) on the wafer of step (vi),

(viii) releasing master structure from said wafer and (optionally) engineering said master structure such as to create the microfluidic chamber, and to cut said master structure to the final form of the master mould,

(ix) use said master structure of step (viii) for the preparation of said polymer chip such as by injection moulding.

A third aspect of the present invention relates to a method for performing a primer extension, said method comprising:

(i) immobilizing a first primer to a detection surface of a solid optical element, wherein said first primer is at least partly complementary, substantially complementary, essentially complementary or complementary to a polynucleotide template,

(ii) contacting a polymerase (DNA polymerase or RNA polymerase) with said first primer and said polynucleotide template in the presence of nucleotides under conditions which allow hybridization of said first primer to said polynucleotide template and extension of said first primer.

A fourth aspect of the present invention provides a kit in parts comprising

(i) a chip comprising at least one solid optical element, preferably a plurality of solid optical elements,

(ii) a first primer as defined in any of the preceding claims, and

(iii) optionally, a second primer as defined in any of the preceding claims.

A further aspect of the present invention relates to the use of the optical chip of the present invention for detecting an analyte.

Brief description of the drawings

Figure 1 shows the setup for the free-angle UV lithography described in details in Example 1.

Figure 2 - Relationship between rotated angle and the effective angle of the final microstructure.

(a) n_a, n_g and n_r are the refractive indexes of air, UV mask glass and resist respectively.

From Snell's law:

$$n_a \sin\theta_{rot} = n_g \sin\theta_1 = n_g \sin\theta_2 = n_r \cos\theta_{SAF}$$

$$\theta_{rot} = \sin^{-1}\left(\frac{n_r}{n_a}\cos\theta_{SAF}\right)$$

In the present case: n_a=1 and n_r=1.582 thus, if θ_{SAF} = 60, θ_{rot} = 52.3

(b) nf = refractive index of fluid (water, air), nt = refractive index of thermopolymer

Figure 3 - SEM image of microfabricated SAF structures by free-angle UV lithography (as described in Example 2). The top surface of the SAF structure was 50 um.

Figure 4 – The relationship between roughness and surface scattering.

Figure 5 – Preparation of a polymer chip, process is in a sequence as following: rotation angle free lithography to create SAF array of either positive or negative tone photoresist, metallization (deposition of a thin metal layer to make electrode for Ni elctrodeposition), Ni Electroplating to produce Ni shim, releasing of photoresist structures from Ni shim by etching the metal layer, Engineering Ni shim to form the final master of the lab-on-a-chip, injection moulding for replication of polymer chip. The fabrication procedures change slightly for positive or negative tone photoresists.

Figure 6 - Illustration of the concept of SP-PCR on SAF micro-optical array. Target specific DNA primers, for example pathogen-specific DNA primers, are directly immobilized on the surface of SAF array located at the bottom of the micro-chamber. PCR reaction mixture is placed in solution with small amount of forward and labelled reverse primers, such as Cy3-labeled reverse primers. The target DNA is initially amplified in the liquid phase to increase the copy number of the starting template. The target DNA then binds to the immobilized primers on the SAF micro-optical array, and the matched primers are extended by the polymerase. By this way, highly-multiplexed amplification can occur in a miniature space with thousands of different primers immobilized at discrete spots. After the reaction, PCR products remain attached to the surface through covalent bonding. The signals are then collected through the SAF structures, and detected using a CCD sensor.

Figure 7 - Possible factors that reduce the yield of amplicons in solid phase amplification. (a) Masking effect. (b) Molecular crowding effect. (c) Neighbouring DNA interaction.

Figure 8 - SP-PCR with different length of primers. S/N of SP-PCR increased linearly as the length of primers.

Figure 9 - The effect of concentration of immobilized primers in SP-PCR. S/N of SP-PCR increased drastically with primer concentration.

Figure 10 - The end type and the centre type of immobilised primers were designed for *hilA*, *fliC* and *sefA* genes of Salmonella spp. This figure is to show that the centre type of the immobilized primer targets central part of the liquid PCR product and the end type primer will result in a shorter 5′ end overhang after SP-PCR.

Figure 11 - Different primers designed within nested liquid phase PCR amplicons of the fliC, sefA, sdf and hilA gene. Centre to end type correspond decreasing the length of 5′ overhang which results in increasing of signal in multiplex solid phase PCR.

Figure 12 - Specificity of a multiplexed SP-PCR for the detection of *Salmonella* spp.

Figure 13 - Sensitivity of the SP-PCR compare to a conventional PCR for the detection of *S. Enteritidis*. Column graph corresponding to S/N of the SP-PCR and the table is to show that the sensitivity of the SP-PCR is comparable to the sensitivity of a conventional PCR measure by bioanalyzer.

Figure 14 – The sensitivity of the SP-PCR compare to a conventional PCR for the detection of *S. Typhimurium*. Column graph corresponding to S/N of the SP-PCR and the table is to show that the sensitivity of SP-PCR is comparable to the sensitivity of the conventional PCR measure by bioanalyzer.

Figure 15 - Image of an injection moulded chip in a microscope slide format. The pitch of the chambers was 9 mm, which was made to fit a multichannel pipette. (b) Image of chambers with integrated SAF arrays. One chamber had a volume of 12 µL, pitch of SAF structures was 0.68 mm. The chamber was surrounded by an energy director for bonding to a COC foil using an ultrasonic welder. (c) SEM image of an injection moulded SAF structure.

Figure 16 - Analytical calculation of light intensity distribution at the air/COC interface. (b) Fluorescent images of 10 µM Cy3-labeled DNA primer taken from (i) the air side and (ii) the COC side. The light ring corresponded to the emission reflected at the side-wall of the SAF structure. (c) The sensitivity of the LOC system. Cy3-labeled DNA primers with concentrations ranging from 2 pM to 20 µM were spotted on the SAF microoptical array and the intensities of the light on the spots were measured.

Figure 17 - Specificity of SP-PCR on the SAF microoptical array. (a) Microarray layout. The DNA primers targeting *hil*A, *sdf*, sefA and *fli*C genes were deposited in 6 consecutive spots for multiplex SP-PCR reaction. The positive control primer was 5'Cy3 –TTT TTT TTT TCC CCC CCC CC– 3' (SEQ ID NO: 1). The UC gene primers for detection of *Campylobacter* spp. was used as a negative control. The SP-PCR was carried out in the microchamber with a volume of 12 µL. (b) Fluorescent image of SP-PCR on the SAF microoptical array to detect *S. Enteritidis*. (c) Fluorescent image of SP-PCR on the SAF microoptical array to detect *S. Typhimurium*.

Figure 18 - Comparison of sensitivity of the SP-PCR amplification performed on the SAF microoptical array and detected on chip vs. those performed on plain COC microarray and detected using a conventional laser scanner. Initial concentrations of S. Enteritidis DNA template varied from 0.15 to 1.5 $\times 10^5$ copies/µL with 10-fold dilution. Signal-to-noise ratios (S/N) of the SP-PCR products for (a) *hil*A, (b) *sdf*, and (c) *sef*A genes were determined separately. The mean value and standard variation were calculated based on

the signals from 6 spots. The lowest template concentration at which positive fluorescence signals could be detected for all the three genes was 1.5 copies/µL.

Figure 19 – Comparison of optical images of an SAF array shim according to the invention manufactured from **A)** AZ125 nXT, **B)** THB151N, and **C)** SU8 showing left over cross-linked photoresist after electroplating with Ni. It was found that whereas hardly any resist was left over after stripping using THB151N, small amounts were left over using AZ125 nXT and significant amounts of resist after using SU8. For both **A)** and **B)**, any resist left over could be removed without damage to the shim by subsequent cleaning using normal means or using the manufacturers' standard removal agents. Significant amounts of SU8 resist were left over as documented in **C)**, which could not be removed without damage to the shim.

Figure 20 – (a) Optical images of the SAF array with 50 µm size (b) 100 µm and (c) 150 µm based on THB 151N photoresist.

Detailed description of the invention

The inventors provide an improved method for solid state PCR by the SP-PCR technology with super critical angle fluorescence (SAF) microoptical array embedded in a microchip. The inventors provided miniaturized SAF microoptical array as part of a microfluidic chamber in thermoplastic material and performed multiplexed SP-PCR directly on top of the SAF array. Attributed to the high fluorescence collection efficiency of the SAF microoptical array, the SP-PCR assay on the LOC platform demonstrated a high sensitivity of 1.5 copies/µl of Salmonella spp, comparable to off-chip detection using conventional laser scanner. The combination of SP-PCR and SAF microoptical array allows for on-chip highly sensitive and multiplexed pathogen detection with low-cost and compact optical components. The LOC platform would be widely used as a high-throughput biosensor to analyze food, clinical and environmental samples.

The inventors found that with the SAF-microoptical array, the limit of detection of the LOC system was 0.8 fluorophores/μm^2. The sensitivity of the SP-PCR assay was determined as low as 1.5 copies/µL of *Salmonella* spp. This detection sensitivity is equal to that obtained by a conventional microarray with data acquisition from a high-end laser scanner. The LOC system presented in this herein work is advantageous in terms of having a small equipment footprint, whilst retaining high sensitivity and multiplexing capability. In addition, incorporation of the SAF microoptical array could be easily realized by injection molding for mass production. The new technologies would result in a portable, high-throughput biosensors that are ideal for online or onsite food safety control, clinical diagnosis as well as environmental monitoring.

The inventors further provide an optical chip comprising one or more solid optical element (SAF structures).

Lab-on-a-chip (LOC) device

Method of preparing an optical chip comprising one or more solid optical element (SAF structures).

An optical chip comprising one or more solid optical element (SAF structures) may be prepared by micro-milling as described in (Hung et al. (2015)). However, the inventors have discovered advantageous properties of optical chip produced by the method of the present invention.

The SAF structures made by micro-milling were relatively big (150 um) and the roughness was high (68 nm).

To further improve the surface quality and enhance the density of SAF array, the inventors have developed a new free-angle UV lithography technique, which together with the injection molding to prepare SAF structures with

smaller dimensions and a reduced surface roughness (the process is outlined in Figure 5).

One aspect of the present invention provides a method for preparing an optical chip according to the invention, said comprising the steps of:

(i) providing a wafer,

(ii) coating said wafer with a photoresist which is not epoxy-based and baking said wafer with said photoresist,

(iii) providing an assembly comprising a fixed light source pointing in the direction of a rotatable holder configured to receive and hold said wafer, and wherein said rotatable holder is configured such that the angle of rotation with respect to said light source may be altered within the range of 0 to 90 degrees, such as 0 to 65 degrees, such 35 to 65 degrees,

(iii) placing said photoresist-coated wafer and a photomask in the rotatable holder such that the photomask covers and is in contact with said wafer,

(iv) exposing said photoresist-coated wafer to a light source capable of developing said photoresist, while the holder rotates with a maximum speed of 4 seconds per turn,

(v) removing uncross-linked photoresist outside of the SAF structures forming on the wafer of step (iv),

(vi) coating the wafer of step (v) with a layer of conducting metal,

(vii) prepare a master structure by electrodeposition of a metal (such as nickel-electrodeposition) on the wafer of step (vi),

(viii) releasing master structure from said wafer and (optionally) engineering said master structure such as to create the microfluidic chamber, and to cut said master structure to the final form of the master mould (for example to create the microfluidic chamber, and to cut the shim to the final form of the master mould),

(ix) use said master structure of step (viii) for the preparation of said polymer chip such as by injection moulding.

In one embodiment a negative photoresist is used, the method comprises the steps of:

(i) providing a wafer in suitable material,

(ii) coating said wafer with a negative photoresist which is not epoxy-based and baking said wafer with said photoresist,

(iii) providing an assembly comprising a fixed light source pointing in the direction of a rotatable holder configured to receive and hold said wafer, and wherein said rotatable holder is configured such that the angle of rotation with respect to said light source may be altered within the range of 0 to below 90 degrees, such as 0 to 65 degrees, such as 35 to 65 degrees,

(iii) Placing said photoresist-coated wafer and a photomask having in the rotatable holder such that the photomask covers and is in contact with said wafer,

(iv) exposing said photoresist-coated wafer to a light source capable of developing said photoresist, with wavelengths in the UV (i-line 365nm) or DUV range, while the holder rotates at a fixed speed (maximum speed of 4 seconds per rotation) ,

(v) removing unexposed photoresist the wafer of step (iv), for example using a resist developer.

(vi) coating said wafer of step (v) with a conducting metal layer, such as Al 500 nm/Au 50 nm, with such as Al 1000 nm/Au 50 nm, with such as Al 1500 nm/Au 50 nm, such as Cr 30 nm/Au 50 nm, such as Ti 30 nm/Au 50 nm, such as NiV 100 nm

(vii) prepare a master structure by electrodeposition of a metal (such as nickel-electrodeposition) on the wafer of step (vi)

(viii) Releasing the said wafer with photoresist structures from master and (optionally) engineering said master structure such as to create the microfluidic chamber, and to cut said master structure to the final form of the master mould,

(ix) use said master structure of step (viii) for the preparation of said polymer chip such as by injection moulding.

The photomask has a pattern of circular holes through which the negative resist is exposed. The holes have a diameter corresponding to the upper diameter of the solid optical element.

Photoresist-coated wafer positioned in the holder is exposed to a light source capable of developing said photoresist, while the holder rotates at a fixed speed sufficient to deliver a dose of light to the wafer and in the same time avoiding the development of the negative resist in the unmasked areas. The speed is preferably not exceeding 4 seconds per rotation. This process step cross-links negative photoresist to the wafer and forms the SAF structures.

In step (v) complete cross-linking resist may be obtained by baking before the unexposed resist outside of the SAF structures formed on the wafer of step is removed, for example using a resist developer.

In one embodiment, the photoresist structures are detached from Ni master by etching said wafer (e.g. silicon wafer) and master in KOH. If the photoresist is not removed in KOH, resist stripping can be performed with stripping products provided by resist manufacturers.

Suitable materials for the wafer are materials with a high glass transition temperature (T_g) and resistance to photoresist developers and polar solvents such as TOPAS 5013. Thus, the wafer may be provided in the form of a polymer material with such properties, glass or silicon. In a preferred embodiment, the wafer is a silicon wafer.

The conducting metal used for the metallization of the wafer may be any metal selected from the list consisting of Al, Cr, Ni, Ti, Au, Pt and combinations thereof such as Al 500 nm/Au 50 nm, with such as Al 1000 nm/Au 50 nm, with such as Al 1500 nm/Au 50 nm, such as Cr 30 nm/Au 50 nm, such as Ti 30 nm/Au 50 nm, such as NiV 100 nm.

An example of assembly comprising a fixed light source pointing in the direction of a rotatable holder configured to receive and hold a wafer is shown in Figure 1.

In another embodiment where a positive-tone photoresist is used steps (i)-(v) is replaced with the following steps:

a. Providing a wafer as previously mentioned, with the sole limitation of substrate transparency to the light used to expose the resist.
b. Coat the wafer with a positive resist and contact it with a photomask with circular holes as the one described previously.
c. Expose the photomask-substrate ensemble in order to deliver the required dose and develop the resist.
d. Deposit a thin metal film in that has the capability of stopping the radiation used to make the template for the SAF structures.
e. Lift-off the resist used for metal patterning.
f. Coat the substrate (used as a mask) with a thin film of positive-tone resist.
g. Expose the coated substrate as in from the backside with a light source similar to the one described in (iv) and capable to enhance the resist solubility in the exposed areas.
h. Develop the substrate in the exposed areas.

If the photoresist is positive the photomask will typically be an array of chromium dots blocking the light where the SAF structures should be. In the case of positive photoresist a transparent wafer with the mask pattern should be used and the photoresist should be exposed through the wafer.

Any suitable photoresist may be used. In one embodiment, the photoresist is a negative photoresist, such as a photoresist selected from the group consisting of, Az 15nXT, AZ 125nXT, JSR negative tone resists THB 111N, THB 126N and THB 151N, preferably THB 151N.

When exposed to the UV light, the negative resist becomes crosslinked/polymerized, and more difficult to dissolve in developer. Therefore, the negative resist remains on the surface of the substrate where it is exposed, and the developer solution removes only the unexposed areas. Masks used for negative photoresists, therefore, contain the inverse or photographic "negative" of the pattern to be transferred.

As detailed in the experiments, epoxy-based photoresists, such as SU8 and related photoresist series, could not detach from the Ni-plated shim mould insert using such stripping means and stripping solutions that would not damage the shim. The cause of this was found to be the highly cross-linked structure of the epoxy-based photoresists.

On the contrary, and as detailed in the experiments, photoresists selected from the group consisting of Az 15nXT, AZ 125nXT, JSR negative tone resists (THB 111N, 126N and 151N), which are not epoxy-based, were found to dissolve readily following normal dissolution procedures or the manufacturers' dissolution solutions, and would provide Ni-plated shim mould inserts with little (AZ-nXT-series) or effectively none (JSR-series) residual photoresist in the shim. In the embodiments of the method for preparing an optical chip, the photoresists of the JSR-series are preferred over the photoresists of the Az-nXT-series with photoresists selected from THB 111N, 126N or 151N being more preferred, and THB 151 being the most preferred photoresist.

In another embodiment, the photoresist is a positive photoresist, such as Az 40XT or Az 9260. In these positive resists, exposure to the UV light changes the chemical structure of the resist so that it becomes more soluble in the developer. The exposed resist is then washed away by the developer solution, leaving windows of the bare underlying material. The mask, therefore, contains an exact copy of the pattern which is to remain on the wafer, as a stencil for subsequent processing.

The tilt angle of the holder may be varied from 0 to 65 degrees, such as 35 to 65 degrees, with respect to the incoming light source. In one embodiment, the tilt angle is 52.3 degrees or about 52.3 degrees.

The tilt angle of the stage for the exposure θ_{rot} is calculated with Snell's law knowing the desired tilt angle of the SAF structures θ_{SAF} as shown in figure 2a. θ_{rot} is the angle of incidence of the light on the resist surface and is linked to θ_{SAF} by the relationship:

$$\theta_{rot} = \sin^{-1}\left(\frac{n_a}{n_r}\cos\theta_{SAF}\right)$$

Where n_a and n_r are the refractive indexes of air and of the resist respectively.

The tilt angle θ_{SAF} depends on the supercritical angle θ_{SC} defined by the theory that regards SAF. θ_{SC} depends intrinsically from the material of which the SAF structure is made and from the medium in which the analyte is suspended through its refractive indices n_s and n_{SAF}.

The optimization of the structures went through the optimization of the angle for total internal reflection. The angle of total internal reflection θ_{tir} depends on the refractive indexes of the materials:

$$\theta_{tir} = f(n_i)$$

The condition for the detection to take place is that the incidence angle of the light on the SAF internal sidewall θ_{inc} is (figure 2b):

$$\theta_{inc} \leq \theta_{tir}$$

While, the optimal detection happens when the light exits the SAF structure perpendicularly to the surface, so that:

$$\theta_{inc} = \frac{\pi}{2} - \theta_{SAF}$$

Since θ_{inc} depends directly on the emission angle θ_{SC}, θ_{SAF} needs to be tuned in such a way that the latter condition applies.

For a SAF structure made out of TOPAS 5013 immersed in water, a value of θ_{SAF} of 60 degrees was calculated for optimal detection.

Any light source capable of cross-linking said photoresist may be used, such as an UV and DUV light source. In one embodiment, the light source is a mercury-vapour lamp or a UV laser.

Using the method of the present invention, it is in possible to make micro-structures at any pre-defined angle. The high-density polymeric SAF array makes it possible to achieve ultra-sensitive multiple-point detection with cheap and compact optical components, and could future expand the use of SAF structures in high-throughput applications.

One aspect of the present invention provides an optical chip comprising at least one solid optical element, wherein said optical element

(i) has a conical frustum shape, defining a lower diameter, an upper diameter, a height, and a surface roughness (RMS roughness), and wherein said lower diameter does not exceed 350 micrometer, said upper diameter does not exceed 175 micrometer, said height does not exceed 150 micrometer, and said surface roughness (RMS roughness) does not exceed 75 nm,

(ii) comprises an upper planar surface defined by said upper diameter to which a detection moiety may be linked, and

wherein the fluorescence emission light entering said optical element at said detection surface at an angle equal to greater than theta(min) is totally internally reflected and (at least substantially) collimated along an axis perpendicular to said detection surface.

By "theta(min)" is meant the minimum angle that the fluorescence emission light incident to the surface of the optical element is capable of entering the optical element and being substantially totally internally reflected such that this fluorescence emission light is substantially collimated and directed toward the detector.

Where the interface is water/glass interface, the refractive indices of water, nw = 1.333, and glass, ng = 1.523, the critical angle amounts to 61.1°.

Fluorescence emission light hitting the upper planar surface at an angle lower than theta(min) is totally reflected on the outer surface of the optical element.

The conical frustum shape of the optical element is also referred to as truncated cones. The conical frustum shape is defined as the frustum created by slicing the top off a right circular cone (with the cut made parallel to the base). It follows that the slant of the conical frustum is straight and the slant height (s) is determined by the following equation (1):

$$s = \sqrt{(R_1 - R_2)^2 + h^2}\,. \qquad (1)$$

where R_1 is the radius of the base and R_2 is the upper planar surface of the conical frustum and h is the height (from the centre of the base to the centre of the upper planar surface).

The optical chip of the present invention is suitable for super critical angle fluorescence (SAF) as described herein and reviewed in Thomas Ruckstuhl and Dorinel Verdes (2004), which reference is incorporated by reference. The optical chip of the present invention has the advantage that the optical element (SAF structure) is smaller in size, which allows for a high density of optical elements on the biochip. The reduced height further reduced the amount a reaction buffer needed for the application, and thus typically requires less sample material. The higher density of optical elements on the biochip also allows for a higher degree of multiplex applications, such as multiplex solid state PCR as described herein.

A further advantage of the solid optical element in form of conical frustum shape over the parabolic shaped elements known in the art is that the preparation of the conical frustum shaped optical element is less complicated.

In a preferred embodiment of the present invention, the optical chip comprises a plurality of said solid optical elements, such as more than 16 solid optical elements, such as more than 20, for example more than 24 solid optical elements, such as more than 28 solid optical elements, for example more than 32 solid optical elements, such as more that 50 solid optical elements, for example more than 100 solid optical elements.

The plurality of said solid optical elements is typically arranged in an array, which may be symmetrical or asymmetrical array depending on the layout of the chip. In one embodiment, the optical chip comprises a symmetrical array comprising at least 4 x 4 solid optical elements, such as at least 6 x 4 solid optical elements.

In one embodiment of the present invention, the height of said optical element is less than 130 micrometer, such as less than 100 micrometer, for examples less 50 micrometer, such as less than 40 micrometer, such as in the range 10-40 micrometer, for example in the range 0.1 – 5 micrometer.

One advantage of reducing the height of the optical elements is that the amount of reaction mixture applied to a micro-chamber comprising the optical elements may be reduced. Further, reduction of the dimensions of the optical elements allows for an increase in the number of optical elements within a given chamber, and thus increasing the multiplexing capability and/or the robustness by having more than one element for detecting of the analyte or a further control.

In one embodiment, the solid optical element or plurality of solid optical elements is positioned in microfluidic chamber. In a further embodiment, the volume of said microfluidic chamber is less than 50 microliter, such as less than 40 microliter, for example less than 30 microliter, such as less than 20 microliter, for example less than 15 microliter or in the range of 5-14 microliter.

The optical element has an upper planar surface, which is also referred to as the detection surface. The shape of the upper planar surface is circular.

In one embodiment, the upper diameter of said optical element (the diameter of the upper planar surface) is less than 150 micrometer, such as less than 100 micrometer, for example less than 50 micrometer, such as in the range of 10-50 micrometer, such as in the range of 0.1 to 5 micrometer.

The optical element has a lower planar surface, which is also referred to as the base. The shape of the base is circular. In a further embodiment, the lower diameter of said optical elements (the diameter of the base) is less than 300 micrometer, such as less than 150 micrometer, for example less than 100 micrometer, such as in the range of 10-100 micrometer , such as in the range of 0.2 to 10 micrometer.

One advantage of reducing the diameters of the optical elements is that the density of optical elements on the chip may be increase.

In a preferred embodiment, the pitch (the distance between the centres of any two neighbouring optical elements) is larger than the lower diameter of the elements to avoid interferences, *i.e.* lower than the diameter of the base of optical element. It follows that when the base diameter is 300 micrometer, the pitch is larger than 300 micrometer. When the base diameter is 150 micrometer, the pitch is larger than 150 micrometer, such as in the range of 300 to 150 micrometer, for example in the range of 250 to 150 micrometer. When the base diameter is 100 micrometer, the pitch is larger than 100 micrometer, such as in the range of 300 to 100 micrometer, for example in the range of 250 to 100 micrometer, such as in the range of 150 to 100 micrometer.

In one embodiment, the optical chip of the invention comprises a plurality of arrays of optical elements. In a further embodiment, the chip comprises a plurality microfluidic chambers each comprising a plurality of optical

elements, which optical elements are preferably arranged in arrays of said optical elements (such as disclosed in Figure 15a and 15b).

Preferably, the chip is made of a high refractive index optical material, such as high refractive index thermal plastics. Preferably, the refractive index of the material is larger than 1.5. Non-limiting examples of such material includes a polymer selected from the list consisting of polystyrene (PS) and/or cyclic olefin copolymer (COC), polycarbonate, polymethyl methacrylate, glycol modified polyethylene terephthalate, cellulose acetate butyrate, PET(polyethylenterephthalat), and MBS (methacrylate-butadiene-styrene).

Typically, the chip comprises a plurality of chambers comprising a plurality of arrays of optical elements, wherein the volume of the chamber could be as small as 2-10 microliter.

The dimensions of the chip may vary according to the format of the reader used for are 25 mm x 76 mm x 1mm (microscope slide format).

For the application of the chip, a detection moiety is attached to upper planar detection surface of at least one of the optical elements. The chip may however be provided without the detection moiety such that the user may select and attach the detection moiety according to the desired application.

In one embodiment of the present invention, the detection moiety is an antibody, protein, a peptide or an antigen. In another preferred embodiment, the detection moiety is an oligonucleotide, microRNA. In one embodiment, the detection moiety is immobilized to the detection surface by a direct chemical linkage. Various forms of suitable oligonucleotides (first and second primers) are described herein. It follows that the chip of the present invention may be provided with a detection moiety in the form of a first and/or second primer as described herein. In one embodiment, the detection moiety is an oligonucleotide, where the size of said oligonucleotide is in the

range of 45 to 80 nucleotides. In another embodiment, the said oligonucleotide is in the range of 1.2 x 10E10 to 2.5 x 10E11 oligonucleotide molecules/mm^2 of said detection surface.

Surface roughness of the SAF structures

Surface roughness of the SAF structures will result the fluorescence emission light lost by scattering effects, especially at the sidewall of the SAF structures where the fluorescent light is collected using total internal reflection. Surface roughness is of paramount importance since the SAF structures are used as optical lens. The amount of scattered light at the surface is directly related to the surface roughness as described in the following equation.

$$\mathrm{TIS}(Rq) = Ro\left[1 - e^{-\left(\frac{4\pi Rq\cos\theta i}{\lambda}\right)^2}\right]$$

- Rq =RMS roughness
- Ro = Theoretical reflectance of the surface
- Θi = The angle of incidence of light on the surface
- λ = the wavelength of the light
- TIS: total integrated scattering

The inventors discovered that the surface roughness of the SAF structure produced by the method of the present invention (free-angle UV lithography) is significantly lower than that of the SAF structure prepare by micro-milling (Example 2 and Figure 4). In particular, the inventors discovered that the surface roughness of the SAF structure produced by the method of the present invention (free-angle UV lithography) was significantly lower than that of the SAF structure prepare by micro-milling, 18 nm vs. 68 nm respectively.

In one embodiment of the present invention, the surface roughness (RMS roughness) of the at least one solid optical element is below 60 nm, such as in the range of 60 to 15 nm, such as below 50 nm, for examples below 40 nm, such as below 30 nm, for example below 20 nm (where λ = 500 nm).

In another embodiment of the present invention, the total integrated scattering (TIS) of said at least one solid optical element is below 0.4, such as in the range of 0.4 and 0.05, such as below 0.3 nm, for example below 0.2, such as below 0,1 nm (where λ = 500 nm).

TIS measurements are made by integrating the Bidirectional Scattering Distribution Function (BSDF) over a portion of the sphere surrounding the scatter source. BSDF is the function that describes the results of measuring optical scattering. The BSDF comes in two flavors, the BRDF for reflection and BTDF for transmission. To measure these functions, one needs a scatterometer, which is an instrument that measures the angular distribution of fluorescence emission light reflected or transmitted by a surface. Several types of scatterometers are available: scanning, Imaging Sphere, conoscope and multiple lens.

Use of Lab-on-a-chip (LOC) device for SP-PCR

A second aspect of the present invention relates to a method for performing a primer extension, said method comprising:

(i) immobilizing a first primer to a detection surface of a solid element, wherein said first primer is at least partly complementary, substantially complementary, essentially complementary or complementary to a polynucleotide template,

(ii) contacting a polymerase (DNA polymerase, other polymerase or RNA polymerase) with said first primer and said polynucleotide template in the presence of nucleotides under conditions which allow hybridization of said first primer to said polynucleotide template and extension of said first primer,

wherein the product obtained in step (ii) comprises at least one fluorescent label, and

(iii) (optionally) illuminating said detection surface of said solid optical element with light at a frequency suitable for exciting said fluorescent label, and

(iv) (optionally) detecting the emission of fluorescent light from said fluorescent label by means of supercritical angle fluorescence detection.

The method of the present invention may be used to amplify and detect polynucleotides (polynucleotide templates) present in sample material.

It follows that the present invention also provides a method for detecting a polynucleotide template, said method comprising:

(i) immobilizing a first primer to a detection surface of a solid element, wherein said first primer is at least partly complementary, substantially complementary, essentially complementary or complementary to a polynucleotide template,

(ii) contacting a polymerase (DNA polymerase, other polymerase or RNA polymerase) with said first primer and said polynucleotide template in the presence of nucleotides under conditions which allow hybridization of said first primer to said polynucleotide template and extension of said first primer,

wherein the product obtained in step (ii) comprises at least one fluorescent label, and

(iii) illuminating said detection surface of said solid optical element with light at a frequency suitable for exciting said fluorescent label, and

(iv) detecting the emission of fluorescent light from said fluorescent label by means of supercritical angle fluorescence detection.

It follows that embodiments described herein for performing a primer extension equally applies to the method for detecting a polynucleotide template described above.

In a preferred embodiment relates to a method for performing a primer extension, said method comprising:

(i) immobilizing a first primer to a detection surface of a solid optical element, wherein said first primer is at least partly complementary, substantially complementary, essentially complementary or complementary to a polynucleotide template,

(ii) contacting a polymerase (DNA polymerase, other polymerase or RNA polymerase) with said first primer and said polynucleotide template in the presence of nucleotides under conditions which allow hybridization of said first primer to said polynucleotide template and extension of said first primer,

wherein the product obtained in step (ii) comprises at least one fluorescent label, and

(iii) (optionally) illuminating said detection surface of said solid optical element with light at a frequency suitable for exciting said fluorescent label, and

(iv) (optionally) detecting the emission of fluorescent light from said fluorescent label by means of supercritical angle fluorescence detection.

Step (ii) may be repeated multiple times, such repetitions are referred to as cycles, such as cycles in a PCR reaction.

The first primer is at least partly complementary, substantially complementary, essentially complementary or complementary to a polynucleotide template. Where the first primer is not complementary to the polynucleotide template, the mismatches between the first primer and the polynucleotide template not prevent the first primer from being extended in the method (step (ii)). Essentially complementary means that the number of mismatching nucleotides is low, for examples 3 or less than 3 mismatching nucleotides in a 20 nt primer.

In one embodiment, sequence of the first primer is more than 85 % sequence identity with the corresponding complementary region of the polynucleotide template, such as more than 90 % or more than 95 % sequence identity with the corresponding complementary region of the polynucleotide template.

The first primer may be immobilized to said detection surface by a direct chemical linkage. Where a second primer is also present, it may also be immobilized to said detection surface by a direct chemical linkage.

In one embodiment, the template is a DNA. In another embodiment, the template is RNA, such as a messenger RNA, microRNA, ribosomal RNA. In a further embodiment, the template is cDNA obtained by reverse transcription of RNA prior to the use of the template by the method of the invention.

It follows that any polymerase capable of extending said first primer hybridized to said polynucleotide template in the presence of nucleotides may be used. The polymerase is preferably a DNA polymerase, such as a thermostable DNA polymerase or reversed transcriptase, for examples a Taq polymerase, phusion polymerase, AmpliTaq Gold polymerase, HotTub polymerase, Tfl polymerase.

In a further embodiment, the method is performed in the presence of a further second primer, for example the first primer being a forward primer and the second primer being a reverse primer for the amplification of the polynucleotide template or a portion thereof.

Thus, in one embodiment, step (ii) is performed in presence of a second primer, wherein said second primer is at least partly complementary, substantially complementary, essentially complementary or complementary to the complementary sequence of said polynucleotide template.

Where the second primer is not complementary to the polynucleotide template, the mismatches between the second primer and the polynucleotide template not prevent the second primer from being extended in the method (step (ii)).

In one embodiment, sequence of the first primer is more than 85 % sequence identity with the corresponding complementary region of the polynucleotide template, such as more than 90 % or more than 95 % sequence identity with the corresponding complementary region of the complementary sequence of said polynucleotide template.

The first and/or second primer may comprise a portion of non-complementary sequence to the target region at the 5′ end of said primer.

In on embodiment, the second primer is immobilized to said detection surface of said solid optical element, preferably solid optical element. This means that both the first primer the second primer, e.g. the forward and the reverser primer, is immobilized to said detection surface.

In a preferred embodiment, the first primer and/or said second primer is an oligonucleotide. In one embodiment, the first primer and/or said second primer are oligonucleotide, which comprises 7 to 25 nucleosides.

The inventors have found that a significant increase in signal to noise ratio (S/N) is obtained when using longer primers from 45 bp to 80 bp. The density of immobilised surface primer was also defined to play a significant role for the S/N of the SP-PCR. The optimum S/N was obtained with surface density of 1.49 × 10E11 molecules/mm^2. In addition, the use of designed nested primer with a short overhang at 5′ helped to improve the S/N of the SP-PCR (Example 3). With the optimized conditions, the inventors successful performed a multiplexed SP-PCR for detection of *Salmonella spp.* at subspecies and for the first time, demonstrated that the SP-PCR can achieve amplification efficiency of 1.5 copies/µl Salmonella spp genome comparable

to a conventional PCR. These improvements will pave the way for wider applications of the SP-PCR in various fields such as clinical diagnosis, high-throughput DNA sequencing and single-nucleotide polymorphism analysis.

Accordingly, in a preferred embodiment, the length of said first and/or said second primer is in the range of 45 to 80 nucleotides. In another preferred embodiment, the density of immobilized first primer and/or second primer is in the range of 1.2 x 10E10 to 2.5 x 10E11 primer molecules/mm^2 of said detection surface. In a further embodiment, the first primer and/or second primer is complementary to a sequence proximal to the 5′ end of said polynucleotide template.

The first primer may be extended and the polynucleotide template amplified by any suitable method of polymerase mediated amplification. In one embodiment, the polynucleotide template is amplified by polymerase chain reaction (PCR) the ligase chain reaction (LCR), loop mediated isothermal amplification (LAMP), nucleic acid sequence based amplification (NASBA), self-sustained sequence replication (3SR), rolling circle amplification (RCA), Strand Displacement Amplification (SDA), Multiple displacement amplification (MDA), Helicase dependant amplification (HAD), Recombinase Polymerase Amplification (RPA), or Nicking Enzyme Amplification Reaction (NEAR).

The extension of the first primer and the amplification of the polynucleotide template are done in the presence of any suitable nucleotides that may be incorporated in a polynucleotide by a polymerase. In a preferred embodiment, the nucleotides are deoxyribonucleotides. In another embodiment, the nucleotides are ribonucleotides. In a further embodiment, the nucleotides are a mixture of deoxyribonucleotides and ribonucleotides. In yet a further embodiment, the nucleotides comprise one or more nucleotide analog(s), such as locked nucleic acid (LNA) or bridged nucleic acid (BNA).

The nucleotides may comprise one or more labelled nucleotide(s). The incorporation of the labelled nucleotide(s) may allow the detection of the

extended primer/amplified template. Thus, nucleotides may comprise one or more labelled nucleotide(s) comprises a fluorescent label.

It follows that where detection relies on fluorescence from a fluorescent label in one of the primers or by extension of the same, the method may comprise the further steps of

(iii) illuminating said detection surface of said solid optical element with light at a frequency suitable for exciting said fluorescent label,

(iv) detecting the emission of light from said fluorescent label by means of supercritical angle fluorescence detection.

Alternatively, one of more nucleosides of the first or second primer may comprise one or more labelled nucleosides. In a preferred embodiment, wherein said one or more labelled nucleosides comprises a fluorescent label.

In a particular embodiment, said first primer comprises a FRET donor and said second primer comprises a FRET acceptor moiety. Alternatively, said first primer comprises a FRET acceptor and said second primer comprises a FRET donor moiety.

Any suitable FRET donor/acceptor pair (referred to as FRET moiety pair) may be used such as but not limited to a FRET acceptor moiety and FRET donor moiety being a FRET moiety pair selected from the group consisting of Cy2/Cy3, Cy3/Cy5, PE/APC, Alexa Flour 488/Alexa Flour 514, Alexa Flour 488/Alexa Flour 532, Alexa Flour 488/Alexa Flour 546, Alexa Flour 488/Alexa Flour 610, fluorescein/Cy5, fluorescein/LC Red 640, fluorescein/LC Red 705, and fluorescein/JA286.

It follows that where a FRET moiety pair is used, the method may comprise the further steps of

(iii) illuminating said detection surface of said solid optical element with light at a frequency suitable for exciting said donor FRET moiety,

(iv) detecting the emission of fluorescent light from said acceptor FRET moiety by means of supercritical angle fluorescence detection.

The buffer is typically performed in a buffer, which is suitable for primer extension. The buffer may be provided including the nucleotides, polymerase and salts as a premix. The template polynucleotide may be mixed with the premix immediately before use. The buffer including all necessary nucleotides, polymerase and salts and the template is referred to as the reaction mixture.

The ratio of primer(s) to polynucleotide may vary. In one embodiment, the ratio of said first primer to polynucleotide template is at least 1.000 to 1, such as least 10.000 to 1, for example 100.000 to 1, such as 1.000.000 to 1. This ration refers to the ratio of said first primer to polynucleotide in the reaction mixture before the first round of application has taken place (step ii in the method of the invention)

In one embodiment, said polynucleotide template is present in a concentration ranging from 1.5 copies of said polynucleotide template per micro litre to 1.5 x 10E5 copies of said polynucleotide template per microliter (of reaction mixture).

The template may any polynucleotide template present in a sample, such as a biological sample. In one embodiment, the polynucleotide template is a polynucleotide obtainable from a microbe or virus. In another embodiment, the polynucleotide template is a polynucleotide obtainable from tissue of a multicellular organism, e.g. a mammal, such as a human being.

As mentioned herein, it is preferred that the method for performing a primer extension is performed using a solid optical element, and thus immobilizing a first primer to a detection surface of a solid optical element (step (i)).

Preferably, the solid optical element forms part of a chip, such as a chip of the present invention. In one embodiment, said chip comprises a plurality of said solid optical elements. It follows that different primers may be immobilized to different solid optical elements, which allows for multiplex analysis of different polynucleotide templates in a sample. Thus, in one embodiment, the first primer immobilized to the detection surface of each of said solid optical elements is not complementary to the same polynucleotide template or complementary to a different portion of the same polynucleotide template. This means that the first primer immobilized to one element and the first primer immobilized to another element does not anneal to same polynucleotide template, or at least not to the same region of the polynucleotide template.

In one embodiment, the chip of the present invention is immersed in a container into a container, such that a reaction buffer comprising nucleotides, template polynucleotides, polymerase and optional further second primer may be added to a micro-chamber of the container such that is covers the surface of the chip. In describing the embodiments of the invention specific terminology will be resorted to for the sake of clarity. However, the invention is not intended to be limited to the specific terms so selected, and it is understood that each specific term includes all technical equivalents which operate in a similar manner to accomplish a similar purpose.

A non-limiting example of the use of a LOC system described herein for solid phase PCR (SP-PCR) is illustrated in Figure 6. The cone-shaped SAF micro-optical array is located at the bottom layout of a micro-chamber. DNA primers specific to different pathogens are immobilized directly on top of the SAF array. The SP-PCR is carried out as Example 3.

Briefly, during initial thermal cycle, if the primers match the DNA sequence in the sample, they will be extended by the DNA polymerase. In the next cycle, the extended primers served as templates for the second strand elongation,

thus generating new templates for the next round amplification. After the reaction, PCR products remain covalently attached to the substrate and fluorescence signals are generated as one of the primers is labelled with fluorescent dye. The signals are then collected through the SAF structures, and the pathogens can be identified from the pattern of fluorescence on the SAF array.

In this example, the SP-PCR reactions for multiplexed detection of Salmonella spp. at subspecies level were developed and performed on the SAF microoptical arrays. Attributed to the low fabrication cost and the ease of integration, the utilization of the SAF array in the SP-PCR detection will allow the SP-PCR to be widely applied for onsite multiplexed pathogen detection.

Kits of parts

A further aspect of the present invention provides a kit in parts comprising

(i) a chip comprising at least one solid optical element, preferably a plurality of solid optical elements,

(ii) a first primer as defined herein, and

(iii) optionally, a second primer as defined herein.

In a preferred embodiment, the optical chip is an optical chip according to the present invention.

Yet, a further aspect of the present invention relates to the use of the optical chip according to the present invention for detecting an analyte, such as by detecting super critical angle fluorescence (also referred to as SAF).

The analyte may be present in any sample. Preferably the analyte is chemical or biological material present in the sample. As described herein, analytes may be detected by use of antibodies, aptamer specifically directed to the analyte. Where the analyte to be detected is a polynucleotide, the

detection moiety is typically an oligonucleotide, such as a first primer as described herein.

In one embodiment, the analyte is selected from the group consisting of a polynucleotide, a polypeptide, an oligopeptide, a virus and a microbe or parts thereof and an organic chemical. The analyte may for example be a pathogenic microbe, virus, hormone, prion, biological substance and chemical agent such as an organic compound. In another embodiment, the analyte is selected from the group consisting of a polynucleotide, a polypeptide, an oligopeptide obtainable from tissue of a multicellular organism, e.g. a mammal, such as a human being.

In one embodiment, a detection moiety capable of binding to said analyte is linked to the detection surface of the solid optical elements of said optical chip, such as by direct chemical or biological linkage. In a further embodiment, the detection moiety is an antibody, which binds said analyte. In a preferred embodiment, the detection moiety is an oligonucleotide, which is elongated using a polymerase, such as a DNA polymerase.

When describing the embodiments of the present invention, the combinations and permutations of all possible embodiments have not been explicitly described. Nevertheless, the mere fact that certain measures are recited in mutually different dependent claims or described in different embodiments does not indicate that a combination of these measures cannot be used to advantage. The present invention envisages all possible combinations and permutations of the described embodiments.

The terms "comprising", "comprise" and "comprises" herein are intended by the inventors to be optionally substitutable with the terms "consisting of", "consist of" and "consists of", respectively, in every instance.

The invention will hereafter be described by way of the following non-limiting items.

Item 1. An optical chip comprising at least one solid optical element, wherein said optical element

(i) has a conical frustum shape, defining a lower diameter, an upper diameter, a height, and a surface roughness (RMS roughness), and wherein said lower diameter does not exceed 350 micrometer, said upper diameter does not exceed 175 micrometer, said height does not exceed 150 micrometer, and said surface roughness (RMS roughness) does not exceed 75 nm,

(ii) comprises an upper planar detection surface defined by said upper diameter to which a detection moiety may be linked, and

wherein fluorescence emission light entering said optical element at said detection surface at an angle equal to or greater than theta(min) is totally internally reflected and (at least substantially) collimated along an axis perpendicular to said detection surface.

Item 2. The optical chip according to item 1, wherein the surface roughness (RMS roughness) of said at least one solid optical element is below 60 nm, such as in the range of 60 to 15 nm, such as below 50 nm, for examples below 40 nm, such as below 30 nm, for example below 20 nm.

Item 3. The optical chip according to any one of items 1 or 2, wherein the total integrated scattering (TIS) of said at least one solid optical element is preferably below 0.4, such as in the range of 0.4 and 0.05, such as below 0.3, for example below 0.2, such as below 0.1, for example bellow 0.05.

Item 4. The optical chip according to any of the preceding items comprising a plurality of said solid optical elements.

Item 5. The optical chip according to any of the preceding items comprising more than 16 solid optical elements, such as more than 20, for example more than 24 solid optical elements, such as more than 28 solid optical elements, for example more than 32 solid optical elements, such as more

that 50 solid optical elements, for example more than 100 solid optical elements.

Item 6. The optical chip according to any of the preceding items, wherein said plurality of said solid optical elements are arranged in an array.

Item 7. The optical chip according to item 6, wherein said array is a symmetrical array comprising at least 4 x 4 solid optical elements, such as at least 6 x 4 solid optical elements.

Item 8. The optical chip according to item 6, wherein said array is an asymmetrical array.

Item 9. The optical chip according to any of the preceding items, wherein the height of said optical element height is less than 130 micrometer, such as less than 100 micrometer, for examples less 50 micrometer, such as less than 40 micrometer, such as in the range 8-43 micrometer, such as in the range 0.1 - 5 micrometer.

Item 10. The optical chip according to any of the preceding items, wherein the upper diameter of said optical element is less than 150 micrometer, such as less than 100 micrometer, for example less than 50 micrometer, such as in the range of 10-50 micrometer, such as in the range 0.1 - 5 micrometer.

Item 11. The optical chip according to any of the preceding items, wherein the lower diameter of said optical elements is less than 300 micrometer, such as less than 150 micrometer, for example less than 100 micrometer, such as in the range of 20-100 micrometer, such as in the range 0.2 - 10 micrometer.

Item 12. The optical chip according to any of the preceding items, wherein the detection moiety is an oligonucleotide.

Item 13. The optical chip according to any of the preceding items, wherein the detection moiety is an antibody.

Item 14. The optical chip according to any of the preceding items, wherein said chip is suitable for detecting super critical angle fluorescence.

Item 15. The optical chip according to any of the preceding items, wherein said chip is made of a high refractive index optical material, such as high refractive index thermal plastics.

Item 16. The optical chip according to any of the preceding items, wherein said chip is made of a polymer selected from the list consisting of polystyrene (PS) and/or cyclic olefin copolymer (COC), polycarbonate, polymethyl methacrylate, glycol modified polyethylene terephthalate, cellulose acetate butyrate, polyethylene terephthalate (PET), and methylmetacrylate butadiene styrene MBS.

Item 17. The optical chip according to any of the preceding items, wherein the dimensions of the optical chip has the format of a standard microscope slide size, such as 76 mm x 26 mm.

Item 18. The optical chip according to any of the preceding items, wherein said solid optical element or plurality of solid optical elements is positioned in microfluidic chamber.

Item 19. The optical chip according to any of the preceding items, wherein the volume of said microfluidic chamber is less than 50 microliter, such as less than 40 microliter, for example less than 30 microliter, such as less than 20 microliter, for example less than 15 microliter, such as less than 10 microliter, for example less than 5 microliter, such as less than 2 microliter, for example less than 500 nanoliter

Item 20. The optical chip according to any of the preceding items comprising a plurality of arrays of optical elements.

Item 21. The optical chip according to any of the preceding items comprising a plurality microfluidic chambers each comprising a plurality of optical elements, which optical elements are preferably arranged in arrays of said optical elements.

Item 22. The optical chip according to any of the preceding items, wherein the distance between the centres of any two neighbouring optical element is larger than the lower diameter of the elements.

Item 23. A method for preparing an optical chip according to any of the preceding items, said method comprising the steps of:

(i) providing a wafer,

(ii) coating said wafer with a photoresist which is not epoxy-based and baking said wafer with said photoresist,

(iii) providing an assembly comprising a fixed light source pointing in the direction of a rotatable holder configured to receive and hold said wafer, and wherein said rotatable holder is configured such that the angle of rotation with respect to said light source may be altered within the range of 0 to 90 degrees, such as 0 to 65 degrees, such as 35 to 65 degrees,

(iii) placing said photoresist-coated wafer and a photomask in the rotatable holder such that the photomask covers and is in contact with said wafer,

(iv) exposing said photoresist-coated wafer to a light source capable of developing said photoresist, while the holder rotates with a fixed speed, preferably maximum speed of 4 seconds per rotation,

(v) removing uncross-linked photoresist outside of the SAF structures forming on the wafer of step (iv),

(vi) coating the wafer of step (v) with a layer of conducting metal,

(vii) prepare a master structure by electroplating (such as nickel-electrodeposition) on the wafer of step (vi),

(viii) releasing master structure from said wafer and (optionally) engineering said master structure such as to create the microfluidic chamber, and to cut said master structure to the final form of the master mould,

(ix) use said master structure of step (viii) for the preparation of said polymer chip such as by injection moulding.

Item 24. The method of any one of item 23, wherein said photoresist is a positive photoresist or a negative photoresist.

Item 25. The method of any one of item 23 or 24, wherein said wafer is silicon, glass or, a thermopolymer, such as a thermopolymer ones with a high glass transition temperature (Tg) and resistance to photoresistpolymer developers and polar solvents for example TOPAS 5013L - 10, for example PS 158 K.

Item 26. The method of any one of item 23 to 25, wherein said conducting metal is gold (Au).

Item 27. The method of any one of items 23 to 26, wherein said a layer of conducting metal is selected from the group consisting of 100 mn Au, a lower layer of 500 nm aluminium (Al) and a upper layer of 50 nm gold Au), a lower layer of 1000 nm aluminium (Al) and a upper layer of 10 nm gold (Au), a lower layer of 1500 nm aluminium (Al) and a upper layer of 50 nm gold (Au).

Item 28. The method of any one of items 23 to 26, wherein said light source is a UV light source or DUV light source.

Item 29. The method of any one of items 23 to 28, said method comprising the steps of:

(i) providing a silicon wafer,

(ii) coating said wafer with a negative photoresist which is not epoxy-based and baking said wafer with said photoresist,

(iii) providing an assembly comprising a fixed light source pointing in the direction of a rotatable holder configured to receive and hold said wafer, and wherein said rotatable holder is configured such that the angle of rotation with respect to said light source may be altered within the range of 0 to 60 degrees,

(iii) placing said photoresist-coated wafer and a photomask having a pattern of dots having a diameter corresponding to the upper diameter of the a solid optical element in the rotatable holder such that the photomask covers and is in contact with said wafer,

(iv) exposing said photoresist-coated wafer to a light source capable of developing said photoresist, such as an UV or DUV light source, while the holder rotates with a fixed speed, preferably at a maximum speed of 4 seconds per rotation,

(v) removing uncross-linkeded photoresist outside of the SAF structures forming on the wafer of step (iv), for example using a resist developer.

(vi) coating the wafer of step (v) with a layer of conducting metal.

(vii) prepare a master structure by electroplating (such as nickel-electrodeposition) on the wafer of step (vi),

(viii) releasing master structure from said wafer and (optionally) engineering said master structure such as to create the microfluidic chamber, and to cut said master structure to the final form of the master mould,

(ix) use said master structure of step (viii) for the preparation of said polymer chip such as by injection moulding.

Item 30. The method according to any one of items 23 to 29, wherein said photoresist is a negative photoresist selected from the group consisting of Az 15nXT, AZ 125nXT, JSR negative tone resists THB 111N, THB 126N and THB

151N, preferably THB 151N, or wherein said photoresist is a positive photoresist selected from the group consisting of Az 40XT, Az 9260 or an image reversal photoresist selected from the group consisting of Az 5214E, TI 35E, AZ nLOF 2020, AZ nLOF 2035, AZ nLOF 2070 and deep UV resist and Az TX 1311.

Item 31. The method according to any one of items 23 to 30, wherein said angle of rotation can be freely adjusted from 0 - 65 degrees, such as 35 to 65 degrees, such as 52.3 degrees or about 52.3 degrees.

Item 32. The method according to any one of items 23 to 31, wherein said light source is i-line (365 nm) or h-line (405 nm) or g-line (436 nm) created by a mercury-vapor lamp or a UV laser combining with a bandpass filter set.

Item 33. A method for performing a primer extension, said method comprising:

(i) immobilizing a first primer to a detection surface of a solid optical element, wherein said first primer is at least partly complementary, substantially complementary, essentially complementary or complementary to a polynucleotide template,

(ii) contacting a polymerase (DNA polymerase or RNA polymerase) with said first primer and said polynucleotide template in the presence of nucleotides under conditions which allow hybridization of said first primer to said polynucleotide template and extension of said first primer,

wherein the product obtained in step (ii) comprises at least one fluorescent label, and

(iii) (optionally) illuminating said detection surface of said solid optical element with light at a frequency suitable for exciting said fluorescent label, and

(iv) (optionally) detecting the emission of fluorescent light from said fluorescent label by means of supercritical angle fluorescence detection.

Item 34. The method for performing a primer extension of item 33, wherein said template is DNA.

Item 35. The method for performing a primer extension of item 33, wherein said template is RNA, such as a messenger RNA, ribosomal RNA or microRNA.

Item 36. The method for performing a primer extension according to any of the preceding items, wherein said DNA polymerase is a thermostable DNA polymerase, such as a Taq polymerase, phusion polymerase, Ampli*Taq* Gold polymerase, Hot*Tub* polymerase, *Tfl* polymerase.

Item 37. The method for performing a primer extension according to any of the preceding items, wherein step (ii) is performed in the presence of a second primer, wherein said second primer is at least partly complementary, substantially complementary, essentially complementary or complementary to the complementary sequence of said polynucleotide template.

Item 38. The method for performing a primer extension according to any of the preceding items, wherein said second primer is immobilized to said detection surface of said solid optical element

Item 39. The method for performing a primer extension according to any of the preceding items, wherein the length of said first and/or said second primer is in the range of 45 to 80 nt.

Item 40. The method for performing a primer extension according to any of the preceding items, wherein the density of immobilized first primer and/or second primer is in the range of 1.2 × 10E10 to 2.5 × 10E11 primer molecules/mm^2 of said detection surface.

Item 41. The method for performing a primer extension according to any of the preceding items, wherein said first primer and/or second primer is

complementary to a sequence proximal to the 5' end of said polynucleotide template.

Item 42. The method for performing a primer extension according to any of the preceding items, wherein said polynucleotide template is amplified by polymerase chain reaction (PCR) the ligase chain reaction (LCR), loop mediated isothermal amplification (LAMP), nucleic acid sequence based amplification (NASBA), self-sustained sequence replication (3SR), rolling circle amplification (RCA), Strand Displacement Amplification (SDA), Multiple displacement amplification (MDA), Helicase dependant amplification (HAD), Recombinase Polymerase Amplification (RPA), or Nicking Enzyme Amplification Reaction (NEAR).

Item 43. The method for performing a primer extension according to any of the preceding items, wherein said nucleotides are deoxyribonucleotides.

Item 44. The method for performing a primer extension according to any of the preceding items, wherein said nucleotides are ribonucleotides.

Item 45. The method for performing a primer extension according to any of the preceding items, wherein said nucleotides is a mixture of deoxyribonucleotides and ribonucleotides.

Item 46. The method for performing a primer extension of item 45, wherein said nucleotides comprise one or more nucleotide analog(s), such as locked nucleic acid (LNA) or bridged nucleic acid (BNA).

Item 47. The method for performing a primer extension according to any of the preceding items, wherein said nucleotides comprise one or more labelled nucleotide(s).

Item 48. The method for performing a primer extension according to any of the preceding items, wherein said nucleotides comprise one or more labelled nucleotide(s) comprises a fluorescent label.

Item 49. The method for performing a primer extension according to any of the preceding items, wherein said first primer and/or said second primer is an oligonucleotide.

Item 50. The method for performing a primer extension according to any of the preceding items, wherein said first primer and/or said second primer is an oligonucleotide comprising 7 to 25 nucleosides.

Item 51. The method for performing a primer extension according to item 50, wherein said nucleosides comprise one or more labelled nucleosides.

Item 52. The method for performing a primer extension according to item 51, wherein said one or more labelled nucleosides comprises a fluorescent label.

Item 53. The method for performing a primer extension according to any one of items 49 to 52, wherein said first primer comprises a FRET donor and said second primer comprises a FRET acceptor moiety.

Item 54. The method for performing a primer extension according to 53, wherein said first primer comprises a FRET acceptor and said second primer comprises a FRET donor moiety.

Item 55. The method for performing a primer extension according to 54, wherein said FRET acceptor moiety and FRET donor moiety is a FRET moiety pair selected from the group consisting of Cy2/Cy3, Cy3/Cy5, PE/APC, Alexa Flour 488/Alexa Flour 514, Alexa Flour 488/Alexa Flour 532, Alexa Flour 488/Alexa Flour 546, Alexa Flour 488/Alexa Flour 610, fluorescein/Cy5, fluorescein/LC Red 640, fluorescein/LC Red 705, and fluorescein/JA286.

Item 56. The method for performing a primer extension according to any of the preceding items, wherein the first and/or second primer comprises a portion of non-complementary sequence to the target region at the 5' end of said primer.

Item 57. The method for performing a primer extension according to any of the preceding items, wherein the first primer is immobilized to said detection surface by a direct chemical linkage.

Item 58. The method for performing a primer extension according to any of the preceding items, wherein the ratio of said first primer to polynucleotide template is at least 1.000 to 1, such as least 10.000 to 1, for example 100.000 to 1, such as 1.000.000 to 1.

Item 59. The method for performing a primer extension according to any of the preceding items, wherein step (ii) is performed in a buffer suitable for primer extension.

Item 60. The method for performing a primer extension according to any of the preceding items, wherein said polynucleotide template is present in a concentration ranging from 1.5 copies of said polynucleotide template per micro litre to 1.5 X 10E5 copies of said polynucleotide template per micro litre

Item 61. The method for performing a primer extension according to any one of the preceding items comprising the further steps of

(iii) illuminating said detection surface of said solid optical element with light at a frequency suitable for exciting said fluorescent label or said donor FRET moiety,

(iv) detecting the emission of fluorescent light from said fluorescent label or said acceptor FRET moiety by means of supercritical angle fluorescence detection.

Item 62. The method for performing a primer extension according to any of the preceding items, wherein said solid optical element forms part of a chip.

Item 63. The method according to any of the preceding items, wherein said chip comprises a plurality of said solid optical elements.

Item 64. The method for performing a primer extension according to item 63, wherein the first primer immobilized to the surface of each of said solid optical elements is not complementary to the same polynucleotide template or complementary to a different portion of the same polynucleotide template.

Item 65. The method for performing a primer extension according to any of the preceding items, wherein said polynucleotide template is a polynucleotide obtainable from a microbe or virus.

Item 66. The method for performing a primer extension according to any of the preceding items, wherein said chip is immersed into a container.

Item 67. A kit in parts comprising

(i) a chip comprising at least one solid optical element, preferably a plurality of solid optical elements,

(ii) a first primer as defined in any of the preceding items, and

(iii) optionally, a second primer as defined in any of the preceding items.

Item 68. The kit of item 67, wherein the optical chip is an optical chip according to any of the preceding items.

Item 69. Use of the optical chip according to any of the preceding items for detecting an analyte.

Item 70. The use according to item 69, wherein a detection moiety capable of binding to said analyte is linked to the detection surface of the solid optical elements of said optical chip.

Item 71. The use according to item 70, wherein the detection moiety is an oligonucleotide, which is elongated using a polymerase, such as a DNA polymerase.

Item 72. The use according to item 70, wherein the detection moiety is an antibody, which binds said analyte.

Item 73. The use according to any of the preceding items, wherein said analyte is selected from the group consisting of a polynucleotide, a polypeptide, an oligopeptide, a virus and a microbe or parts thereof.

Item 74. The use according to any of the preceding items, wherein said analyte is selected from the group consisting of a pathogenic microbe, virus, hormone, prion, biological substance and chemical agent such as an organic compound.

Item 75. The use according to any of the preceding items, wherein said analyte is detected by detecting super critical angle fluorescence.

Examples

Example 1 – Method of preparing an optical chip comprising solid optical elements

An optical chip comprising solid optical elements (SAF structures) was prepared as follows. A negative photoresist AZ125nxt was used to coat the silicon wafer. After baking, the substrate together with the photomask (containing pattern of dots with diameter corresponding to the top surface of the miniaturized SAF structure) was put into a holder. The holder and setup

is shown in Figure 1. The setup consists of a fixed UV source and a rotating holder that holds a photomask and a photoresist-coated substrate together. The holder can rotate with the speed of maximum 4 sec/ turn (15 rpm), and the angle can varied from 0 to 60 degrees. Four screws were used to ensure firm contact of the mask and substrate. The method is referred herein as free-angle UV lithography.

In exposure process, the holder is tilted and rotated to a UV source. The relationship between rotated angle and the effective angle of the final microstructure was calculated as shown in Figure 2. In order to get a 60 degree side wall for the SAF structure, the rotation angle was determined to be 52.3 degree.

After UV exposure, the substrate was developed in resist developer. As shown in Figure 3, the 3-dimensional cone-shaped structures were obtained on the silicon wafer. An example of the process by process steps is shown in Figure 5.

Example 2 - Characterization of surface roughness

To characterize the roughness of the sidewall surface, the inventors have engineered a holder with a slope angle of 60 degrees and then mounted a piece of the injection moulded SAF structure on this surface to level the sidewall relative to the instrument. The roughness of the SAF structures was characterized using a PLu Neox 3D optical profiler (Sensofar, USA), an optical profilometer, with a vertical resolution of less than 2 nm (as described in Hung et al. (2015).

The inventors discovered that the surface roughness of the SAF structure produced by the method of the present invention (free-angle UV lithography) was significantly lower than that of the SAF structure prepare by micro-milling, 18 nm vs. 68 nm respectively. As one can see and read from Figure 4 (and the algorithm on which it is based), with roughness of 68 nm, the

percent of scattered light is 45%; while with roughness of 18 nm, the percent of scattered light is reduced to 8%. The results imply that SAF structures fabricated by method of the present invention can provide much better optical quality.

Example 3 – Solid Phase PCR

***Salmonella* DNA extraction**

Salmonella Enteritidis (CCUG 92243) and *Salmonella* Typhimurium (Jeo 3979 Jgt.110) were used in this study. Bacterial cultures were grown at 37°C for 18 h in Buffered Peptone Water (BPW). One mL of the culture was collected and centrifuged at 15,000 × g for 5 min at 5°C. The pellet was washed using 1 mL phosphate buffered saline (PBS, Oxoid). Bacterial chromosomal DNA was extracted from the pellet using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The DNA concentration was determined by Nano drop (Thermo Scientific, USA). The extracted DNA was stored at -20°C until use. By considering the genome size of *Salmonella* approximately 4.8 Mb, the genome equivalence can be calculated as below equation and were used for calculating the limit of detection of SP- PCR.

Number of copies = $\frac{AB}{CDE}$

A = Amount of DNA in nanogram.

B = Avogadro's number which is 6.022×10^{23}.

C= Genome size of *Salmonella* which is 4.8 Mb.

D = Multiply by 1×10^{9} to convert to nanograms

E= Assumption that the average weight of a base pair (bp) is 650 Daltons.

Primer design

PCR primers were designed for specific detection of *Salmonella* spp. at subtype level. A Nucleotide-BLAST from NCBI was used to test for homology

within the database and Primer-BLAST for primer designed. Four different primer sets targeting 4 different genes: *the hil*A (Genbank access no. U25352), *sdf* (Genbank access no. AF370707.1), *sef*A (Genbank access no. L11008.1) *and fli*C (Genbank access no. EF599295.1) of Salmonella spp were designed to specifically identify different *Salmonella genus*. The *hil*A gene is specific for *Salmonella genus* while the *sdf* gene is specific for serotypes *Salmonella* Enteritidis; the *sef*A gene is specific for serotypes *Salmonella* Dublin and *Salmonella* Gallinarum; and the *fli*C gene is specific for *Salmonella* Typhimurium and *Salmonella* Kentucky. A primer set namely UC originating from *16S* rRNA gene of *Campylobacter* (Genbank access no. GQ249182.1) was used as a negative control. For each gene, one pair of a forward and a Cy5 labelled reverse primer was designed for the liquid phase amplification and a nested primer was designed as solid attached support primer. To enhance the amplification efficiency of the SP-PCR, the melting temperatures of the reverse primers were calculated around 3.2 - 9°C higher than those of the forward primers. All the primers were synthesized and purchased by DNA technology (Aarhus, Denmark). The primers sequences are shown in Table 1.

Table 1 list of primers and primers used in this study

Species	gene	PCR primers' sequences (5'-3') and TM	Product size
Salmonella spp	*hilA*	*hilA* -F: GCGACGCGGAAGTTAACGAAG A (SEQ ID NO: 2), Tm 62.2 *hilA* –R Cy5-CACGATAGAGTAATGCAGACTCTCGGATTGAACCTGATC (SEQ ID NO: 3), Tm 66.1 hilA-primer TTTTTTTTTTCCCCCCCCCCCGGTTTAATCGTCCGGTCGTA	225 bp

GTGGTGTCTCCGCCAGCGCCGCAACCTACGACTCATACA (SEQ ID NO: 4), Tm Without poly TC tails = 79.2, With poly TC tails = 81.8

S.Enteritidis, S.Dublin and *S.Gallinarum*	*sefA*	sefA-F: GTGGTTCAGGCAGCAGTTACT (SEQ ID NO: 5), Tm 58.9 sefA-R: Cy5- TGTGACAGGGACATTTAGCGTTTCTTGAG (SEQ ID NO: 6), Tm 62.1 sefA-primer TTTTTTTTTTCCCCCCCCCCCGTATTCAGGGAGCCAATATTAATGACCAAGCAAATACTGGAATTGACGGGCTTGCAGGT(SEQ ID NO: 7), Tm Without poly TC tails = 72.6, With poly TC tails = 76.8	334 bp
Salmonella enteritidis	*sdf*	sdf-F: AAATGTGTTTTATCTGATGCAAGA GG (SEQ ID NO: 8), Tm 54.7 sdf-R: Cy5-TCTAATGAACTACGTTCGTTCTTCTGGTACTTACGATGAC (SEQ ID NO: 9), Tm 63.7 sdf-primer TTTTTTTTTTCCCCCCCCCCATCAAAAAGGTTTAGTAAATCAGCCTGTTGTCTGCTCACCATTCGCCAGCCACCACCTTC (SEQ ID NO: 10), Tm Without poly TC tails = 73.8, With poly TC tails = 77.3	299 bp
S.Typhimurium and *S.Kentucky*	*fliC*	fliC-F: CCCCGCTTACAGGTGGACTAC (SEQ ID NO: 11), Tm 61.9 fliC-R: CTGCAGCGGGTTTTCGGTGGTTGT (SEQ ID NO: 12), Tm 66.7 fliC-primer TTTTTTTTTCCCCCCCCCCACTTACGCTGCAAGTAAAGCC	433 bp

		GAAGGTCACAACTTTAAAGCACAGCCTGATCTGGCGGAA (SEQ ID NO: 13), Tm Without poly TC tails = 75.1 With poly TC tails = 78.2
Campylobacter spp.	*16S rRNA*	UC-primer TTTTTTTTTCCCCCCCCCCAGGAAGGTGTGGACGACGTC AAGTCATCATGGCCCTTATGCCCAGGGCGACACACGTGCT (SEQ ID NO: 14), Tm Without poly TC tails = 80.2 With poly TC tails = 82.1

Immobilization of primer on COC polymer

A simple UV cross-linking method for attaching TC-tagged DNA oligonucleotides on various substrates has previously described (Sun et al., 2012), The technique has been showed not only high versatility but also high thermal stability comparable to other tedious and expensive covalent bonding methods. In this study, this method was used to immobilize the primer on COC polymer. COC slide (Microfluidic ChipShop, Germany) was cleaned in 70% ethanol in sonication for 30 minutes then rinsed with Milli-Q water (Millipore Corporation, USA) and air dried. The five oligonucleotide primers with poly(T)10–poly(C)10 tails (Table 1) were diluted in 5 × saline sodium citrate (SSC) buffer (Promega, WI, USA) with 0.004% Triton X-100 (Sigma-Aldrich, USA). The primers solutions were spotted on the clean COC substrate using a non-contact array nano-plotter 2.1 (GeSim, Dresden, Germany). Each primer was spotted in six consecutive spots for confirmation of the results. After the drying, the slides were exposed to UV irradiation at 254 nm with energy of 0.3 J cm^2 (Stratalinker 2400, Stratagene, CA, USA) to immobilize the primers onto surface of the substrate. The slide was washed in 0.1 × SSC solutions for 5 min, then rinsed in deionized water and dried at 25°C. The slides were treated with BSA (2.5 mg/mL) for 30 minutes and rinsed with Milli-Q water and air dried before use.

Solid phase PCR reaction

A 25 µL of SP-PCR reaction mixture was prepared. The SP-PCR mixture consists of 1 × Phusion® Human Specimen PCR Buffer (Thermo Fisher

Scientific), 400 nM of *hilA* forward and 1600 nM *hilA* reverse primers, 200 nM of *sdf* forward and 800 nM *sdf* reverse primers, 200 nM of *sefA* forward and 800 nM *sef*A reverse primer, 600 nM of *fliC* forward and 2400 nM *fliC* reverse primer, 400 ng bovine serum albumin (BSA) (Sigma- Aldrich St. Louis, USA) and 0.05 U/µL Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Fisher Scientific). A Gene Frame (Thermo Fisher Scientific) was used to create a 25 µL chamber surrounding the primer array. The PCR master mix was pipetted into the gene frame and sealed with a cover slip. The SP-PCR was conducted in a flat-bed PCR machine (MJ research, Canada). As suggested for enhancing the SP-PCR increased annealing temperature was used during later PCR cycles. The SP-PCR conditions were: 94°C for 5 minutes and 30 cycles of 94°C for 10 seconds, 60°C for 20 seconds and 72°C for 20 seconds, followed by another 15 PCR cycles of 94°C for 10 seconds, 65°C for 20 seconds and, 72°C for 20 seconds. After the SP-PCR, the chamber was washed with 0.1 × SSC and 0.1% of Sodium dodecyl sulphate (SDS) (Promega, WI, USA) for 5 minutes then rinsed with deionized water and dried at room temperature.

Data analysis

The slides were scanned using BioAnalyzer 4F/4S equipped with a light scanner (LaVision BioTech GmbH, Bielefeld, Germany). Microarray image was analyzed by GenePix pro 7.0 software (Molecular devices). A circle adjusted to the size of the spot and determined the intensity values per spot and perimeter of a square surrounding the circle were the background signal. The signal to noise ratio (S/N) was defined as the mean of signal intensity of the feature, subtracting the mean of background signal, and dividing by the variation in the background. Experiments were performed in duplicate and the *UC* primers were used as internal control (Table 1).

Optimization of solid-phase PCR

The effect of primer length

Different lengths of the *hil*A primer ranging from 45 bp to 80 bp were designed (Table S1). Sixty (60) µM of each primers was spotted on COC

substrates to test the effect of the primer length. The slides were then subjected to SP-PCR reaction as described.

The effect of primer density

Different concentrations of *hil*A primer (80 bp) ranging from 5 μM to 100 μM were spotted on COC. The volume of the droplet deposited by Nano plotter was approximately 0.06 nL, and the size of the spot on COC was 100 μm in diameter. The immobilization efficiency of UV-crosslinking method is around 50%, so the actual primer density is from $1.24x\ 10^{10}$ to $2.48\ x\ 10^{11}$ molecules/mm^2. The SP-PCR reaction was carried out with the same condition as described.

The effect of primer annealing position

For the *hil*A, *fli*C and *sef*A genes, two types of primers were designed to target different positions of the liquid phase PCR amplicon products (Table S2). The primers have the same length of 80 bp, but varied in the distance to 5′ end of the liquid PCR amplicon products. All primers were spotted at the same concentration of 60 μM and tested using SP-PCR.

Specificity and sensitivity of SP-PCR

After optimization of the length of primers, concentration and annealing position, four primers targeting *hil*A, *sdf, sef*A *and fli*C genes were spotted on the COC slides for multiplex SP-PCR reaction. Genomic DNA of *S.* Enteritidis and *S.* Typhimurium were used to investigate the specificity of the developed SP-PCR. A series of 10-fold dilution ranging from 1.5×10^5 copies/μl to 1.5×10^{-1} copies/μl were employed for sensitivity test.

For comparison, a conventional liquid-phase PCR was also conducted on PCR machine (MJ Research PTC-200) with the same PCR condition as the SP-PCR. The PCR amplified products were quantified on Agilent 2100 BioAnalyzer (Palo Alto, CA, USA) using DNA 12000 chip.

Involvement of immobilised solid support primers in SP-PCR

The percentage of the immobilised solid support primers involved in the SP-PCR was determined by using a standard curve. Serial dilutions of control primer with Cy5 label ranging from 0.06 µM to 60 µM were spotted on COC slide. After UV crosslinking and washing, the S/N was measured to build a standard curve. In theory, the fluorescence intensity of the 60 µM spot represents the case when 100% of the immobilized primers are extended during SP-PCR. A "Trend" function in Microsoft Excel (Microsoft Office Excel 2010) was used to fit the S/N of the *hil*A, *sdf*, *sef*A and *fli*C genes signals obtained after the SP-PCR to the standard curve. The involvement of the immobilised solid support primers in SP-PCR was calculated by the equation

$$\frac{\mathrm{a}}{60\ \mathrm{es}} \times 100\ \% = \mathrm{b}$$

a = Primer concentration obtained by fitting S/N to the standard curve.

b = Percentage of solid support primers involved during SP-PCR.

RESULTS AND DISCUSSION

The effect of length of primer

It has been shown that longer primers increased signal intensity in microarray hybridization. However, to the inventor's knowledge no work has been done to study the effect of length of primers on SP-PCR. The inventors designed *hilA* gene primers with different lengths and spotted on COC slide at concentration of 60 µM. As shown in Figure 8, the signal to noise ratio (S/N) increased with the primer length from 45 bp to 80 bp. The experimental results indicated that long DNA primer gave a more intense amplification signal than the short one. The findings are in agreement with those reported in literature. For DNA hybridization, Chou et al. (2004) showed that a length of 150 bp was the optimal primer length for expression measurement. The normalized intensity of microarray hybridization at 63 ºC between 100 bp and 50 bp of primers was approximately 2.6 fold

increments. A similar results was observed in SP-PCR, when the inventor compare the use of the two primers with the length of 45 bp and 80 bp the S/N of the SP-PCR reduce approximately 8 fold.

In SP-PCR, long primer facilitates the hybridization of solution PCR products. As the primer length increases, more complementary binding sites are available for the target. Thus, the longer primers have a higher probability than the shorter primers to interact with the target, which implies that longer primers can provide higher sensitivity. However increasing the primer length may also address a number of limits in solid phase amplification. Longer primers can alleviate the masking effect. Since after immobilization to the surface, the primers are expected to extend further away from the immobilised surface. This will make the primers more accessible to DNA targets and this contributes to good conditions for DNA polymerase e to efficient annealing and extension.

The effect of surface primer density

To investigate the effect of surface primer density, the SP-PCR was carried out on COC slides containing 80 bp *hil*A primer with the surface density ranging from 1.24×10^{10} to 2.48×10^{11} molecules/mm^2. The correlation between the S/N and the primer density is shown in Figure 9. Less signal was observed when the primer density was below 4.96×10^{10} molecules/mm^2. The fluorescence intensity increased significantly when the density exceeded 9.92×10^{10} molecules/mm^2 and the maximum S/N was obtained at density of 1.49×10^{11} molecules/mm^2. However, the signal started to decline with further increase of surface density.

The results suggested that within a certain range, amplification may be limited by insufficient primers, so that higher surface density resulting from higher concentration of spotted primers largely improved the surface amplification efficiency. However, above a certain limit, the high amount of the immobilized primer tends to inhibit the amplification. The soundest explanation is that the molecular crowding effect plays an important role in

the SP-PCR at high primer density. When the primers are separated by less than the radius of gyration, the steric effect from neighbouring primers will result in a repulsive force on the targets. That raises the difficulty for the liquid phase PCR products to anneal to the immobilised surface probes. In theory when the length of the primer after extension reaching 112 bp. The SP-PCR extention product will has a theoretical gyration radius of 3 nm. In order to prevent this, the primer distance should be larger than the gyration radius and therefore the density of the primers should, approximately be $1.1x10^{11}$ molecules per mm^2. The theoretical number fits well to the $1.49x10^{11}$ molecules/mm^2 as determined experimentally (corresponding to the spotting concentration of 60 μM). The optimized surface density was employed for further experiments to archive efficient SP-PCR DNA amplification on the surface with minimized molecular crowding effect.

Effect of primer position in DNA target

In order to examine the effect of annealing position of the primers on the targets, two types of primers were designed for *hil*A, *fli*C and *sef*A genes: the end type and the center type primers (Figure 10). The red strand and blue strand represent the immobilized primer and primers from liquid phase, respectively. The center-type primer targets the central part of the liquid PCR amplicon product, whereas the end-type primer is allocated closer to the 5'end of the DNA target, resulting in a shorter 5' end overhang. The SP-PCR results showed that the fluorescence signal increased from 3 to 19 fold for all the three genes when using end-type primers.The results showed that the extention of the annealed primer and the DNA target is highest when the primer is located closer to the 5' end of the target (Figure 11).

Peytavi *et al.(2005)* observed a similar effect in microarray hybridization. This phenomenon could be explained by the free energy related to the hybridization reaction between the primer and target. In SP-PCR, in the case of the 80 bp primer,the number of nucleotides bound to the target is the same for both end and center types probes, the enthalpy is equal in all the

annealing reactions. Hence the free energy is affected only by the entropy term. However for the end-type probe, the loss in configurational entropy upon the annealing is less than in the case of the center type primer, thus it has a higher opportunity for annealing all its sequences to the target.

In addition, in the end-type primer the effect of neighboring DNA interactions is limited since when the target anneals to the end-type primer, the shortened 5'end overhang mades it more difficult for the target to interfere with an adjacent amplicon. Therefore, the inhibitory effect due to neighboring DNA interactions is less likely to occur, which leads to a higher yield of surface amplicons in the end type primer.

Sensitivity and efficiency of the multiplexed SP-PCR

With the optimized conditions, a multiplexed SP-PCR was performed to detect *Salmonella* spp. as well as identify Salmonella the serotypes. Four 80 bp end-type probes for *hil*A, *sdf*, *sef*A and *fli*C genes were spotted on COC slides with a density of 1.49×10^{11} molecules/mm^2. The layout of the array is shown in Figure 12a. The *hil*A, *sdf* and *sef*A genes are used to detect *S. Enteritidis* and the *fliC* genes is used to differentiate S. Typhimurium. The SP-PCR was conducted with DNA extracts from *S. Enteritidis* and *S. Typhimurium*. As shown in Figure 12b, the two *Salmonella* strains were accurately identified by the distinct patterns of PCR amplified products. No positive signal was obtained for the *Campylobacter* UC negative control primer, and no cross amplification was observed, showing that the primers were highly specific.

The sensitivity of the SP-PCR for *hil*A, *sdf*, *sef*A and *fli*C genes were determined and compared with a conventional PCR (Figure 13 and Figure 14). The fluorescence signal of the SP-PCR array increased with template concentration, suggesting that more amplification occurred on the surface at higher template concentration. It was also noticed that there is a high variation in S/N among different genes. As suggested by Khan *et al*.(year),

this phenomenon was owing to the inherent difference in hybridization efficiencies between oligonucleotides. Despite of the variation, remarkably high sensitivities were achieved for all the four genes as summarized in Table 2. The lowest DNA concentration able to be detected by the SP-PCR on COC slide was determined as low as 1.5 copies/µl for both *S. Enteritidis* and *S. Typhimurium*, which was the same as conventional PCR. The percentage of solid support primers involved in the SP-PCR was calculated and listed in Table 2. Approximately 44.9% - 77.2% of the immobilized primers were extended during the solid phase amplification, which was significantly higher than the 36% as previously reported (Palanisamy et al.,2010). The results showed that by addressing all the issues that affect the yield of the surface amplification, the SP-PCR efficiency can be improved enormously.

Table 2. The efficiency of multiplex SP-PCR on glass and COC targeting *hil*A, *sdf* and *sef*A genes.

Gene	Percentage of immobilized primers involved in SP-PCR	
	S. Enteritidis	*S. Typhimurium*
hilA	69%	61.2%
sdf	74.8%	-
sefA	77.2%	-
fliC	-	44.9%

Conclusion

The inventors have tested and characterized the length of the immobilized primer, the surface density of immobilized primer and the annealing position of the primers in targets that theoretical influence the efficiency of the SP-

PCR. The theoretical hypotheses were confirmed experimentally by multiplex SP-PCR performed on COC substrate for detection and identification of food borne pathogens *Salmonella* spp. at sub species level. The best S/N was obtained with end-type 80 bp primers at surface density of 1.49×10^{11} molecules/mm^2. With the optimized conditions, the detection limit of 1.5 copies/µl was obtained and up to 77% of surface primers were involved in the surface amplification. To the inventor's knowledge, this study presents the first evidence that SP-PCR can achieve high sensitivity that is comparable to conventional PCR. This work provided guidelines for developing efficient DNA amplification on a solid surface, which would greatly facilitate the applications of SP-PCR in fields such multiplexed diagnostics, next generation sequencing and high-throughput screening.

Example 4 - Fabrication of injection moulded chip with SAF microoptical array

The microchip with dimension of a microscope slide (76 mm × 25 mm × 1 mm) was fabricated in cyclic polyolefin copolymer (COC) (TOPAS 5013-10, Topas Advanced Polymers GmbH, Germany) by injection molding (Engel Victory 80/45 Tech, PA, USA). To make the injection molding insert, a computer controlled micro-milling system was used (Folken Ind., Glendale, California, USA). The inverted master of unichip integrating microfluidic chambers and SAF arrays was milled using a 60° engraving tip (DIXI 7006, Le Locle, Switzerland) in hard aluminium (alloy 2017, MetalCentret, Denmark). The surface was followed by polishing to smooth the master. As shown in Figure 15, the polymeric chip had eight chambers located parallel at the centre of the chip with a pitch of 9 mm. Each chamber had dimension of 5 mm (length) × 3.6 mm (width) × 0.4 mm (height), corresponding to a volume of 12 µL. In the middle of each chamber was a miniaturized SAF microoptical array of 32 truncated cone-shape structures (diameter of top surface 150 µm; diameter of rear surface 300 µm; height 130 µm and pitch 680 µm). Microfluidic channels with width of 350 µm and depth of 400 µm were connected the chambers for sample processing. The surface

roughness of the SAF structure was qualified by a PLu Neox 3D optical profiler (Sensorfar, USA).

Chemicals

Chemicals and reagents used in this study were of analytical grade purchased from Pierce Inc., USA and Sigma-Aldrich, USA unless otherwise specified.

Bacterial strains and culture conditions

Salmonella reference strains *Salmonella* Enteritidis (CCUG 92243) and *Salmonella* Typhimurium (Jeo 3979 Jgt.110) were from a strain collection of National Food Institute, Technical University of Denmark (DTU-Food). The *Sal. Enteritidis* reference strain was originated from a culture collection of the university of Goteborg. The strains were resuscitated and selected on Xylose Lysine Desoxycholate Agar (XLD) and grown overnight on Blood Agar (BA, 40 g/L of blood agar base no.2 (CM271, Oxoid, Basingstoke, UK) supplemented with 5 % calf blood at 37 °C before use.

DNA preparation

Bacterial stock was grown at 37 °C for 18 h in Buffered Peptone Water (BPW). One ml of the bacteria mixture in BPW was collected and centrifuged at 15,000× g for 5 min at 5 °C. The pellet was suspended in 1 mL phosphate buffered saline (PBS, Oxoid) by vortex and centrifuges again in 15,000× g for 5 min at 5°C. The bacterial pellet was subjected to the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The extracted DNA was stored at -20 °C. DNA concentration was determined by Nano drop (Thermo Scientific, USA).

Primers and primers

PCR primers and primers were designed for specific detection of *Salmonella* spp. at subtype level. Four different genes were selected to specifically identify different *Salmonella genus*. The *hil*A gene is specific for *Salmonella genus* while *sdf* gene is specific for serotypes *Salmonella* Enteritidis; *sef*A gene is specific for serotypes *Salmonella* Dublin and Gallinarum*; fliC* gene is specific for *Salmonella* Typhimurium and Kentucky. A Campylobacter universal primer namely UC primer set that was originated from 16S Ribosomal DNA gene of *Campylobacter* genus was used as a negative control. For each gene, one pair of forward and Cy3 labelled reverse primers was designed for the liquid phase amplification and one nested primer was designed as solid support primer. The primer was modified at the 5' end with a poly (T)10-poly (C)10 tail to facilitate the attachment to the plastic substrates. All of the primers and primers were synthesized and purchased by DNA technology (Aarhus, Denmark) and the sequences are listed in Table 3.

Table 3. List of primers and primers used in this study.

Species	gene	PCR primers' sequences (5'-3')

Salmonella spp.	*hilA*	hilA -F: GCGACGCGGAAGTTAACGAAG A (SEQ ID NO: 2) hilA –R Cy3- CACGATAGAGTAATGCAGACTCTCGGATTGAACCTGATC (SEQ ID NO: 3) hilA-primer TTTTTTTTTTCCCCCCCCCCAAGAGCATCGTTACATTGAAAC ACTGTACGGACAGGGCTATCGGTTTAATCGTCCGGTCG (SEQ ID NO: 15)
S.Enteritidis, S.Dublin and *S.Gallinarum*	*sefA*	sefA-F: GTGGTTCAGGCAGCAGTTACT (SEQ ID NO: 5) sefA-R: Cy3-TGTGACAGGGACATTTAGCGTTTCTTGAG (SEQ ID NO: 6) sefA-primer TTTTTTTTTTCCCCCCCCCCCGTATTCAGGGAGCCAATATTA ATGACCAAGCAAATACTGGAATTGACGGGCTTGCAGGT (SEQ ID NO: 7)
S. Enteritidis	*sdf*	sdf-F: AAATGTGTTTTATCTGATGCAAGA GG (SEQ ID NO: 8) sdf-R: Cy3- TCTAATGAACTACGTTCGTTCTTCTGGTACTTACGATGAC (SEQ ID NO: 9) sdf-primer TTTTTTTTTTCCCCCCCCCCCATCAAAAAGGTTTAGTAAATCA GCCTGTTGTCTGCTCACCATTCGCCAGCCACCACCTTC (SEQ ID NO: 10)
S.Typhimurium and *S.Kentucky*	*fliC*	fliC-F: CCCCGCTTACAGGTGGACTAC (SEQ ID NO: 11) fliC-R: Cy3-CTGCAGCGGGTTTTCGGTGGTTGT (SEQ ID NO: 12) fliC-primer TTTTTTTTTTCCCCCCCCCCCACTTACGCTGCAAGTAAAGCCG AAGGTCACAACTTTAAAGCACAGCCTGATCTGGCGGAA (SEQ ID NO: 13)

Campylobacter spp.	*16S rRNA*	UC-primer TTTTTTTTTTCCCCCCCCCCAGGAAGGTGTGGACGACGTCA AGTCATCATGGCCCTTATGCCCAGGGCGACACACGTGCT (SEQ ID NO: 14)

Immobilization of DNA primers on SAF microoptical array

A simple UV cross-linking technique was used to direct immobilize the poly(T)poly(C)-tagged DNA oligonucleotide primers on the SAF array without any surface modification (Sun et al., 2012). The primers were diluted in 150 mM sodium phosphate buffer (pH 8.5) containing 0.004 % Triton X to a final concentration of 60-80 µM. The DNA primers were deposited on top of the SAF array using a non-contact nano-plotter 2.1 (GeSim, Dresden, Germany). The Picoliter pin injected 60 pL/droplet and the spot size was around 100 µm. Each primer was spotted in six consecutive spots for confirmation of the results. After drying the spots, the microchip was exposed to an UV irradiation at the wavelength of 254 nm with a power of 3 mW/cm^2 for 10 min (Stratalinker 2400, Stragtagene, CA, USA). Subsequently, the chip was washed in 0.1× standard saline citrate (SSC) with 0.1 % (w/v) sodium dodecyl sulfate (SDS) (Promega, WI, USA) solution, then gentle rinsed in deionized water and dried by nitrogen.

SP-PCR on SAF microoptical array

The optimized condition for SP-PCR described in Example 3 was employed in this study. After immobilization of DNA primer at optimized concentration, The microchip was bonded with a 254 µm-thin COC film using an ultrasonic bonder (USP 4700, Techsonic, Herstad-Piber, Denmark) at trigger force of 750 N, energy of 70 Ws and holding time of 0.35 s. Twelve µL of PCR master mixture was then loaded into each chamber through the inlet hole. The master mix contained DNA template, 1 × Phusion® Human Specimen PCR Buffer (Thermo Fisher Scientific), 400 nM of *hila* forward and 1600 nM *hil*A reverse primers, 200 nM of *sdf* forward and 800 nM *sdf* reverse primers, 200 nM of *sef*A forward and 800 nM *sef*A reverse primer, 600 nM of *fli*C forward and 2400 nM *fli*C reverse primer, 16 ng/µl bovine serum albumin (BSA) and

0.125 U Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Fisher Scientific). After loading, the inlet and outlet holes were sealed by PCR compatible tape (Applied Biosystems, USA). The SP-PCR was carried out using a bench-top thermal cycler (Thermo Scientific, USA) at 94 °C for 5 min then followed by 30 cycles of 94 °C for 10 sec, 60 °C for 20 sec and 72 °C for 20 sec, and another 15 cycles of 94 °C for 10 sec, 65 °C for 20 sec and 72 °C for 20 sec. After reaction, the microchambers were washed 3 times by 0.1 % SDS and 0.1× SSC buffer solution followed by MQ water.

Image acquisition and data analysis

A compact optical module was built for detection of fluorescent dye Cy3 in SP-PCR on SAF micro array. This module included a green LED array light source (525 nm, Thorlabs, NJ, USA), a cy3 filter cube (excitation 513-556 nm, emission 570-613 nm, dichroic cut-on wavelength 562 nm, Edmund Optics, UK) and an USB digital microscope (ScopeEye, NKS Tech ApS, Denmark). Working principle of this optical system can be summarized as follows: Cy 3 fluorophores were excited by the green LED light after passing through the excitation filter 513-556 nm and the 45º dichroic mirror. The emission light of Cy3 fluorophores after collection by the SAF array was filtered by the dichroic mirror and the emission filter 570-613 nm. Then the emission light was recorded and imaging by the USB digital microscope. Fluorescence intensity was quantified using ImageJ software. A circle was adjusted to the size of the spot and the mean value of grey levels of the pixels inside a fluorescent spot was calculated. A square was drawn surrounding the circle, and the mean signal was taken as background. The signal to noise ratio (S/N) in this study was defined as the mean signal intensity of the feature subtracted by the mean background, and divided by the variation of the background.

Results and discussion

Characterization of the SAF microoptical array

SP-PCR efficiency on different substrates was investigated in Example 3. It was found that the SP-PCR efficiency was highest on COC in comparison to

glass and polystyrene. Thus, in this example, COC was used substrate material to fabricate the SAF microoptical arrays.

Based on the theories of SAF emission proposed by Ruckstuhl et al (2000), the angular emission distribution is anisotropic for a surface-bound fluorescent molecule. The distribution of light intensity of fluorophores sitting at an air-COC interface was calculated and illustrated in Figure 16a. It shows that a significant emission maximum around the direction of the critical angle of total internal reflection, which is given by θ = arcsin (1/1.53) = 40°. Using the known angular distribution of the fluorescence emission, the dimensions of the microoptical elements were carefully designed. The design principle was explained in details in a previous study (Hung et al. 2015). Parameters considered include height, angle of the cone, as well as inner and outer diameter. The SAF structure with 60° angle was chosen to ensure total internal reflection at the side wall.

The high-precision micro-milling and polishing provided low surface roughness down to 60 nm, which warranted the high-quality optical property of the SAF microoptical array. The scattering light at the sloped wall of the SAF element due to the surface roughness was estimated from Equation 1 as

$$P_{\text{scat}} = \left(1 - e^{-\left(\frac{4\pi\sigma\cos\theta_i}{\lambda}\right)^2}\right) P_{\text{tot}} \quad (1)$$

where P_{tot} is the total flux of incident light, P_{scat} is the flux of light scattered, λ is the wavelength, θ_i is the incident angle, and σ is the surface roughness (Su et al., 2005). Assuming that the scattering light accounts for the major optical power loss, the insertion loss caused by the surface roughness was approximately 0.25 dB at the wavelength of 525 nm.

Detection limit of the integrated LOC system

To know the light collection efficiency of the SAF structure made in COC, 10 μM Cy3-labeled DNA primer was spotted on the SAF microoptical array. Fluorescent images were taken and compared their intensities from both the top (air side) and the bottom (COC side) of the SAF microoptical array. As shown in Figure 16b, when taking the image directly from the top, only a

small dot could be seen; while for the image taken from bottom, a brighter dot surrounded by a light ring as a "donut" was observed and the signal increased approximately 40 folds. The light ring corresponded to the fluorescence emission reflected at the side-wall of the SAF structure.

To determine the sensitivity of the system, Cy3-labeled primer was spotted at concentrations ranging from 2 pM to 20 μM on the SAF microoptical array. Intensity of the fluorescent light measured with the SAF structure is plotted *vs.* primer concentration shown in Figure 16c. The detection limit was 0.2 nM, corresponding to 0.8 fluorophores per μm^2, whereas the detection limit of the signal measured without the SAF structure using the same optical system was determined to be 8 nM, corresponding to 32 fluorophores per μm^2. Thus, the SAF structures enhanced the sensitivity of the system by 40 folds.

In a conventional optical detection, it is common to collect surface-generated fluorescence from the air side. However, as demonstrated here, the luminescence collection efficiency was poor since only a small amount of surface fluorescence went into air, and it may be subject to further optical loss depending on the numeric aperture of the lens (Hung et al, 2015). In contrast, substantial enhancement in fluorescence collection efficiency was achieved with the SAF microoptical array, and the low detection limit clearly indicated the high sensitivity of the SAF microoptical array system. Moreover, the miniaturized dimension of the microoptical array allows the benefits of SAF microscopy to be fully utilized in multiplexed detection.

Specificity and sensitivity of SP-PCR assay on SAF microoptical array

Four DNA primers targeting *hil*A, *sdf, sef*A *and fli*C genes were spotted on the SAF microoptical array for multiplex SP-PCR reaction. The layout of the array is shown in Figure 17a. Genomic DNA of *S.* Enteritidis and *S.* Typhimurium were used to investigate the specificity of the SP-PCR assay. The distinct fluorescence patterns were shown in Figs. 17a and 17b, indicating that the two *Salmonella* strains could be accurately identified based on the specific combinations of amplified products. No fluorescence signal was obtained for

the UC *Campylobacter* control primer. In particular, unspecific amplification was almost negligible as the primers in SP-PCR were spatially separated, showing that the assay was highly specific. In addition, the throughput of the SP-PCR can easily be increased by further expanding the size of the SAF microoptical array, offering the flexibility of interrogating targets ranging from a few to hundreds or even thousands.

The sensitivity of the SP-PCR on the SAF microoptical array was tested using 10-fold dilution series of *S.* Enteritidis genomic DNA ranging from to 0.15 copies/µL to 1.5×10^5 copies/µL. For comparison, the same SP-PCR reactions were carried out on a microarray on plain COC substrates. In this later case, fluorescence signals were detected using a conventional laser scanner (LaVision BioTech GmbH, Bielefeld, Germany).

As shown in Figure 18, the signal-to-noise ratio (S/N) of the on-chip multiplexed SP-PCR for *hilA*, *sdf*, and *sefA* genes were determined separately. The lowest template concentration at which positive fluorescence signals could be detected for all three genes was 1.5 copies/µL. The S/N increased with template concentration, implying that more amplification occurred on the surface at higher amount of initial template. The signals reached plateau when the DNA template was present at 1.5×10^4 copies/µL, indicating the surface became saturated with the PCR products. The coefficient of variation of the replicate reactions was 9 % on average. It was also noticed that the S/N varied a lot among different genes. As suggested by Khan *et al.*, this was owing to the inherent difference in hybridization efficiencies between oligonucleotides (Khan *et al.*, 2008). Despite of the variation, high sensitivities were achieved for all of the three genes. When compared to the conventional SP-PCR that utilized laser scanner to measure fluorescent signals, the LOC system with the SAF microoptical array showed an identical sensitivity and linear range though the S/Ns obtained were generally lower. The results suggested that highly sensitive and multi-point detection could be achieved on chip without the use of benchtop sensor systems. As high-density SAF microoptical arrays can be easily fabricated by injection molding, they are readily to be incorporated into low-cost and

portable microchip devices. The excellent light collection power of SAF microoptical array and high specificity and multiplexing capability of the SP-PCR will be a perfect combination for high-throughput detection in point-of-care settings.

Conclusions

In conclusion, the present LOC platform that allowed the performance of multiplexed SP-PCR on an injection-molded polymeric SAF microoptical array. The enhanced fluorescence collection efficiency and the miniaturized dimension of the SAF structures enabled highly sensitive and multi-point optical detection on chip. Using an inexpensive and handheld optical module for measuring fluorescent signals, detection limit of 0.8 fluorophores per μm^2 was achieved. The SP-PCR reactions developed for multiplexed detection of *Salmonella* spp. at subspecies level were performed on the SAF microoptical arrays in microfluidic chambers. The minimum detectable target concentration was 1.5 copies/µL, which was comparable to those obtained using conventional microarray scanner. As the LOC platform is easy to fabricate and exhibits significant analytical performance features, it has great potential to become a basic tool for point-of-care applications for parallel analysis of molecular interactions, such as gene expression, pathogen detection or single nucleotide polymorphism (SNP) analysis.

Example 5 - Fabrication of injection moulded chip with SAF microoptical array – comparison of photoresists Injection moulded chips were manufactured according to the procedure of Example 1 using **A)** AZ125 nXT, **B)** THB151N, and **C)** SU8. After stripping optical images were recorded of the shims and the results compared to determine the suitability of the specific photoresist in the method of the invention.

Figure 19 displays a comparison of optical images of an SAF array shim according to the invention manufactured from **A)** AZ125 nXT, **B)** THB151N, and **C)** SU8 showing left over cross-linked photoresist after electroplating with Ni. It was found that whereas hardly any resist was left over after stripping using THB151N, small amounts were left over using AZ125 nXT and

significant amounts of resist after using SU8. For both **A)** and **B)**, any resist left over could be removed without damage to the shim by subsequent cleaning using normal means or using the manufacturers' standard removal agents. Significant amounts of SU8 resist were left over as documented in **C)**, which could not be removed without damage to the shim.

Figure 20 displays (a) Optical images of the SAF array with 50 µm size (b) 100 µm and (c) 150 µm based on THB 151N photoresist, documenting that also at these sizes of the optical elements of the invention, THB 151N was a suitable photoresist in the method of the invention.

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Claims

1. An optical chip comprising at least one solid optical element, wherein said optical element

(i) has a conical frustum shape defining a lower diameter, an upper diameter, a height, and a surface roughness (RMS roughness), and wherein said lower diameter does not exceed 350 micrometer, said upper diameter does not exceed 175 micrometer, said height does not exceed 150 micrometer, and said surface roughness (RMS roughness) does not exceed 75 nm,

(ii) comprises an upper planar detection surface defined by said upper diameter to which a detection moiety in the form of an oligonucleotide or an antibody may be linked, and

wherein fluorescence emission light entering said optical element at said detection surface at an angle equal to or greater than theta(min) is totally internally reflected and (at least substantially) collimated along an axis perpendicular to said detection surface.

2. The optical chip according to claim 1, wherein the surface roughness (RMS roughness) of said at least one solid optical element is below 60 nm, such as in the range of 60 to 15 nm, such as below 50 nm, for examples below 40 nm, such as below 30 nm, for example below 20 nm.

3. The optical chip according to any one of claims 1 or 2, wherein the total integrated scattering (TIS) of said at least one solid optical element is preferably below 0.4, such as in the range of 0.4 and 0.05, such as below 0.3, for example below 0.2, such as below 0.1, for example bellow 0.05.

4. The optical chip according to any of the preceding claims comprising a plurality of said solid optical elements, preferably comprising more than 16 solid optical elements, such as more than 20, for example more than 24 solid optical elements, such as more than 28 solid optical elements, for example

more than 32 solid optical elements, such as more that 50 solid optical elements, for example more than 100 solid optical elements.

5. The optical chip according to any of the preceding claims, wherein the height of said optical element height is less than 130 micrometer, such as less than 100 micrometer, for examples less 50 micrometer, such as less than 40 micrometer, such as in the range 8-43 micrometer, such as in the range 1 - 5 micrometer.

6. The optical chip according to any of the preceding claims, wherein the upper diameter of said optical element is less than 150 micrometer, such as less than 100 micrometer, for example less than 50 micrometer, such as in the range of 10-50 micrometer, such as in the range 1 - 5 micrometer.

7. The optical chip according to any of the preceding claims, wherein the lower diameter of said optical elements is less than 300 micrometer, such as less than 150 micrometer, for example less than 100 micrometer, such as in the range of 20-100 micrometer, such as in the range 1 - 10 micrometer.

8. The optical chip according to any of the preceding claims, wherein the distance between the centres of any two neighbouring optical element is larger than the lower diameter of the elements.

9. A method for preparing an optical chip according to any of the preceding claims, said method comprising the steps of:

(i) providing a wafer,

(ii) coating said wafer with a photoresist which is not epoxy-based and baking said wafer with said photoresist,

(iii) providing an assembly comprising a fixed light UV light source or DUV light source pointing in the direction of a rotatable holder configured to receive and hold said wafer, and wherein said rotatable holder is configured such that the angle of rotation with respect to said light source

may be altered within the range of 0 to 90 degrees, such as 0 to 65 degrees, such as 35 to 65 degrees,

(iii) placing said photoresist-coated wafer and a photomask in the rotatable holder such that the photomask covers and is in contact with said wafer,

(iv) exposing said photoresist-coated wafer to a light source capable of developing said photoresist, while the holder rotates with a fixed speed, preferably maximum speed of 4 seconds per rotation,

(v) removing uncross-linked photoresist outside of the SAF structures forming on the wafer of step (iv),

(vi) coating the wafer of step (v) with a layer of conducting metal,

(vii) prepare a master structure by electroplating (such as nickel-electrodeposition) on the wafer of step (vi),

(viii) releasing master structure from said wafer and (optionally) engineering said master structure such as to create the microfluidic chamber, and to cut said master structure to the final form of the master mould,

(ix) use said master structure of step (viii) for the preparation of said polymer chip such as by injection moulding.

10. The method according to claim 9, said method comprising the steps of:

(i) providing a silicon wafer,

(ii) coating said wafer with a negative photoresist which is not epoxy-based and baking said wafer with said photoresist,

(iii) providing an assembly comprising a fixed light source pointing in the direction of a rotatable holder configured to receive and hold said wafer, and wherein said rotatable holder is configured such that the angle of rotation with respect to said light source may be altered within the range of 0 to 60 degrees, such as 35 to 65 degrees,

(iii) placing said photoresist-coated wafer and a photomask having a pattern of dots having a diameter corresponding to the upper

diameter of the a solid optical element in the rotatable holder such that the photomask covers and is in contact with said wafer,

(iv) exposing said photoresist-coated wafer to a light source capable of developing said photoresist, such as an UV or DUV light source, while the holder rotates with a fixed speed, preferably at a maximum speed of 4 seconds per rotation,

(v) removing uncross-linked photoresist outside of the SAF structures forming on the wafer of step (iv), for example using a resist developer.

(vi) coating the wafer of step (v) with a layer of conducting metal.

(vii) prepare a master structure by electroplating, such as nickel-electrodeposition, on the wafer of step (vi),

(viii) releasing master structure from said wafer and (optionally) engineering said master structure such as to create the microfluidic chamber, and to cut said master structure to the final form of the master mould,

(ix) use said master structure of step (viii) for the preparation of said polymer chip such as by injection moulding.

11. The method according to any of the claims 9 or 10, wherein said photoresist is chosen from the group comprising Az 15nXT, AZ 125nXT, and JSR negative tone resists THB 111N, THB 126N, or THB 151N, preferably THB 151N.

12. A method for performing a primer extension, said method comprising:

(i) immobilizing a first primer to a detection surface of a solid optical element according to any one of the preceding claims, wherein said first primer is at least partly complementary, or complementary to a polynucleotide template,

(ii) contacting a DNA polymerase or RNA polymerase with said first primer and said polynucleotide template in the presence of nucleotides

under conditions which allow hybridization of said first primer to said polynucleotide template and extension of said first primer,

wherein the product obtained in step (ii) comprises at least one fluorescent label.

13. The method for performing a primer extension according to any of the preceding claims, wherein the length of said first primer is in the range of 45 to 80 nt.

14. The method for performing a primer extension according to any of the preceding claims, wherein the density of immobilized first primer is in the range of 1.2 × 10E10 to 2.5 × 10E11 primer molecules/mm^2 of said detection surface.

15. A kit in parts comprising

(i) a chip comprising at least one solid optical element according to any of the preceding claims, preferably a plurality of said solid optical elements,

(ii) a first primer as defined in any of the preceding claims, and

(iii) optionally, a second primer.

16. Use of the optical chip according to any of the preceding claims for detecting an analyte, wherein a detection moiety capable of binding to said analyte is linked to the detection surface of the solid optical elements of said optical chip,

wherein the detection moiety is an oligonucleotide, which is elongated using a polymerase, such as a DNA polymerase or an antibody, which binds said analyte,

wherein said analyte is selected from the group consisting of a polynucleotide, a polypeptide, an oligopeptide, a virus or a microbe or parts thereof, hormone, prion, biological substance and chemical agent such as an organic compound.

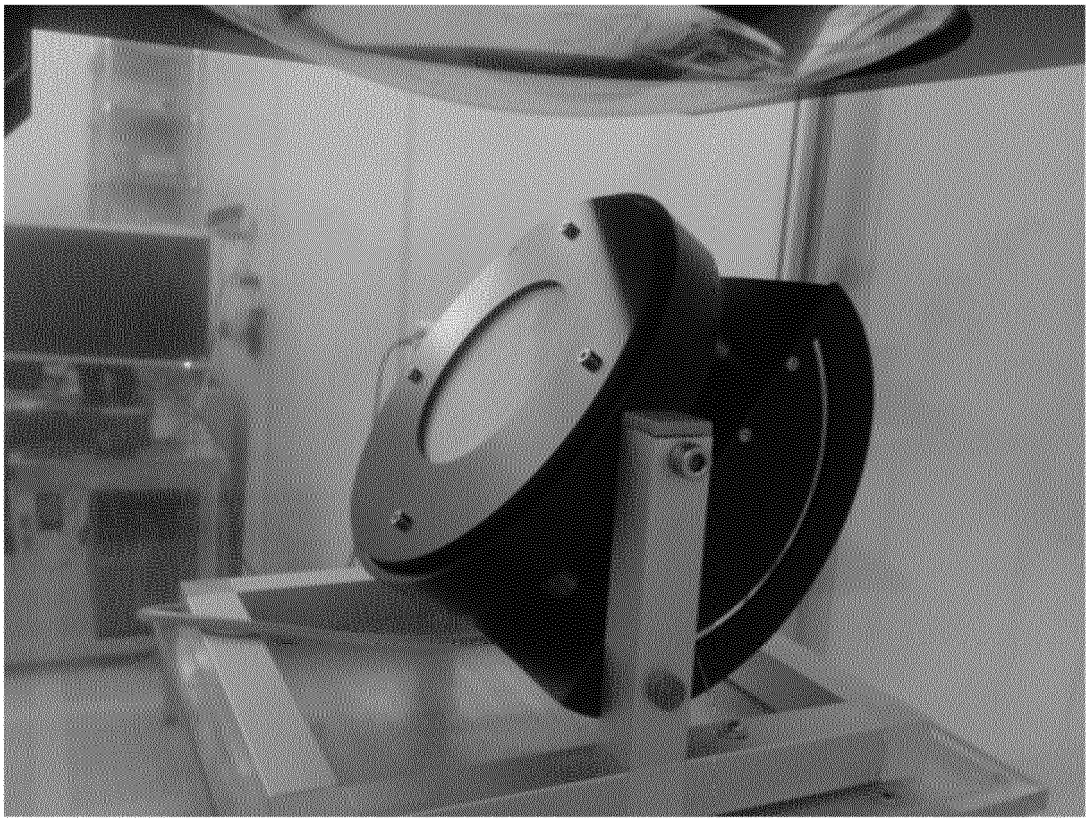


Figure 1

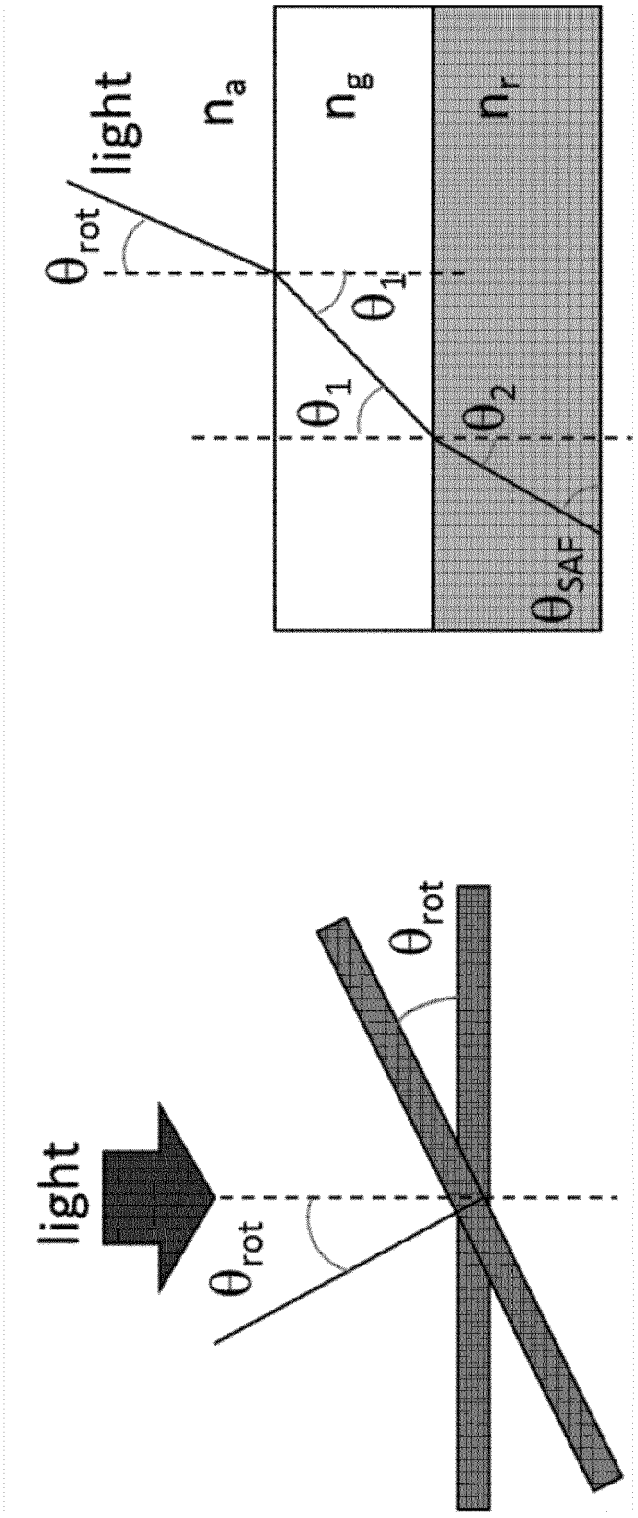

light
n_a
n_{gg}
n_r
θ_{rot}
$\theta_{1'}$
θ_1
θ_2
θ_{SAF}
light
θ_{rot}
θ_{rot}

Figure 2A

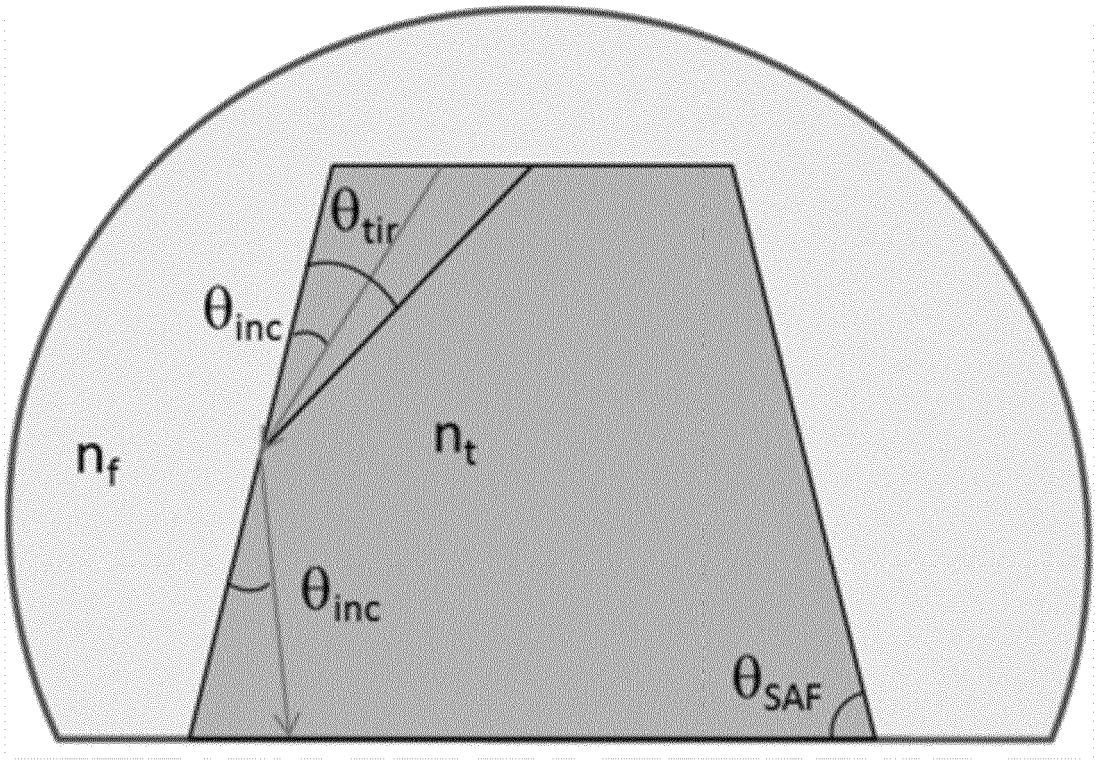


Figure 2B

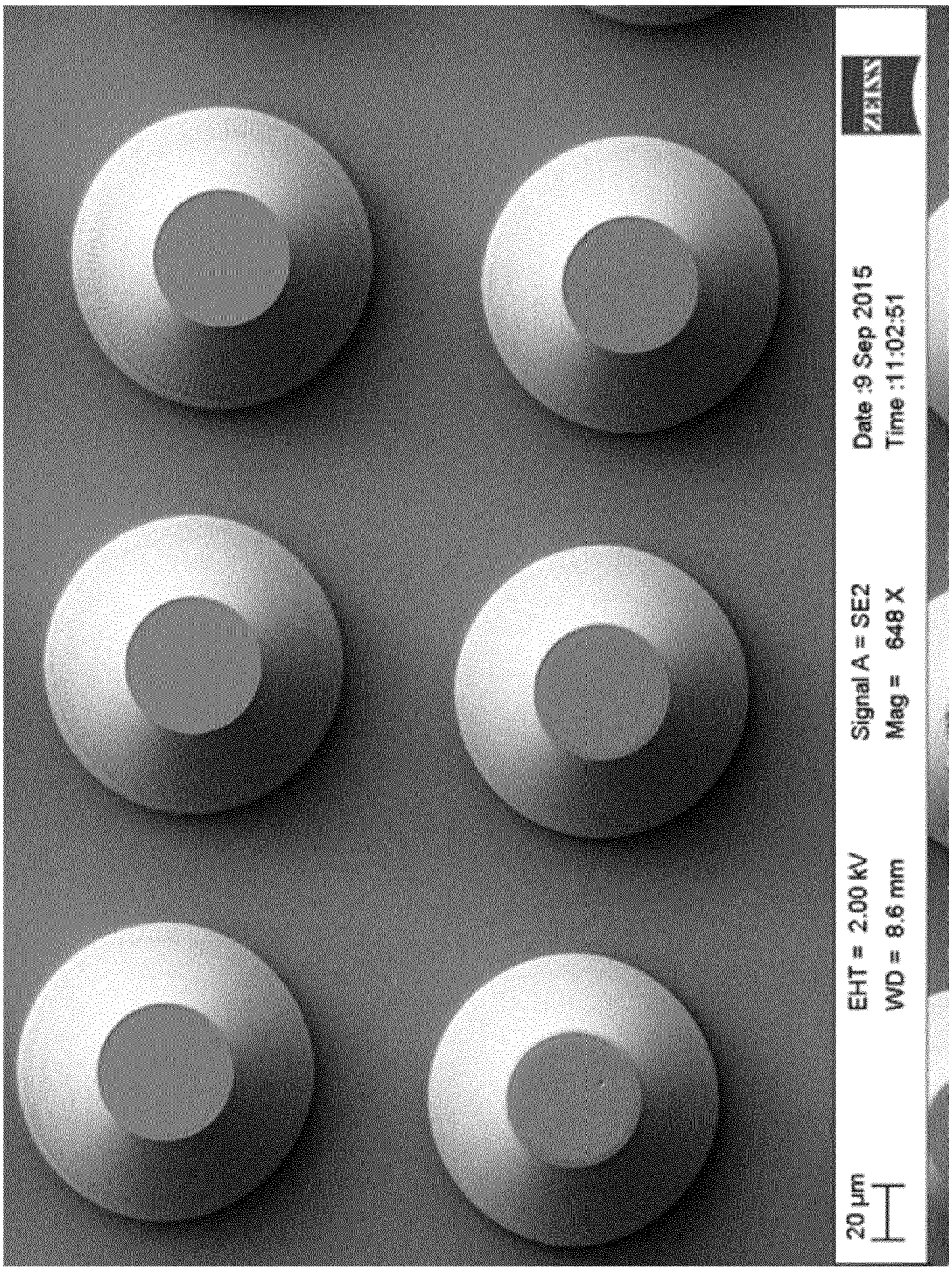

20 µm
EHT = 2.00 kV
WD = 8.6 mm
Signal A = SE2
Mag = 648 X
Date :9 Sep 2015
Time :11:02:51
ZEISS

Figure 3

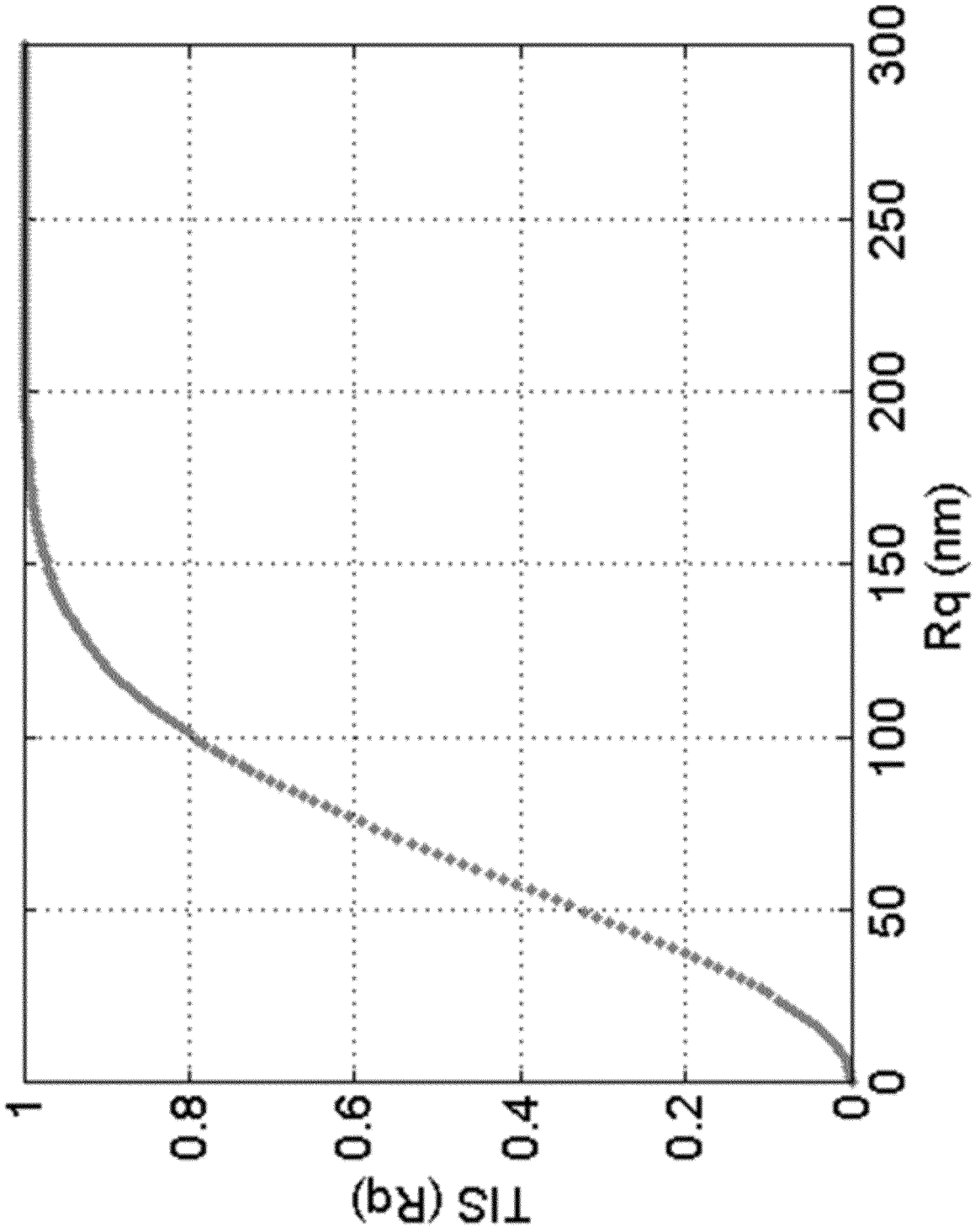


Figure 4

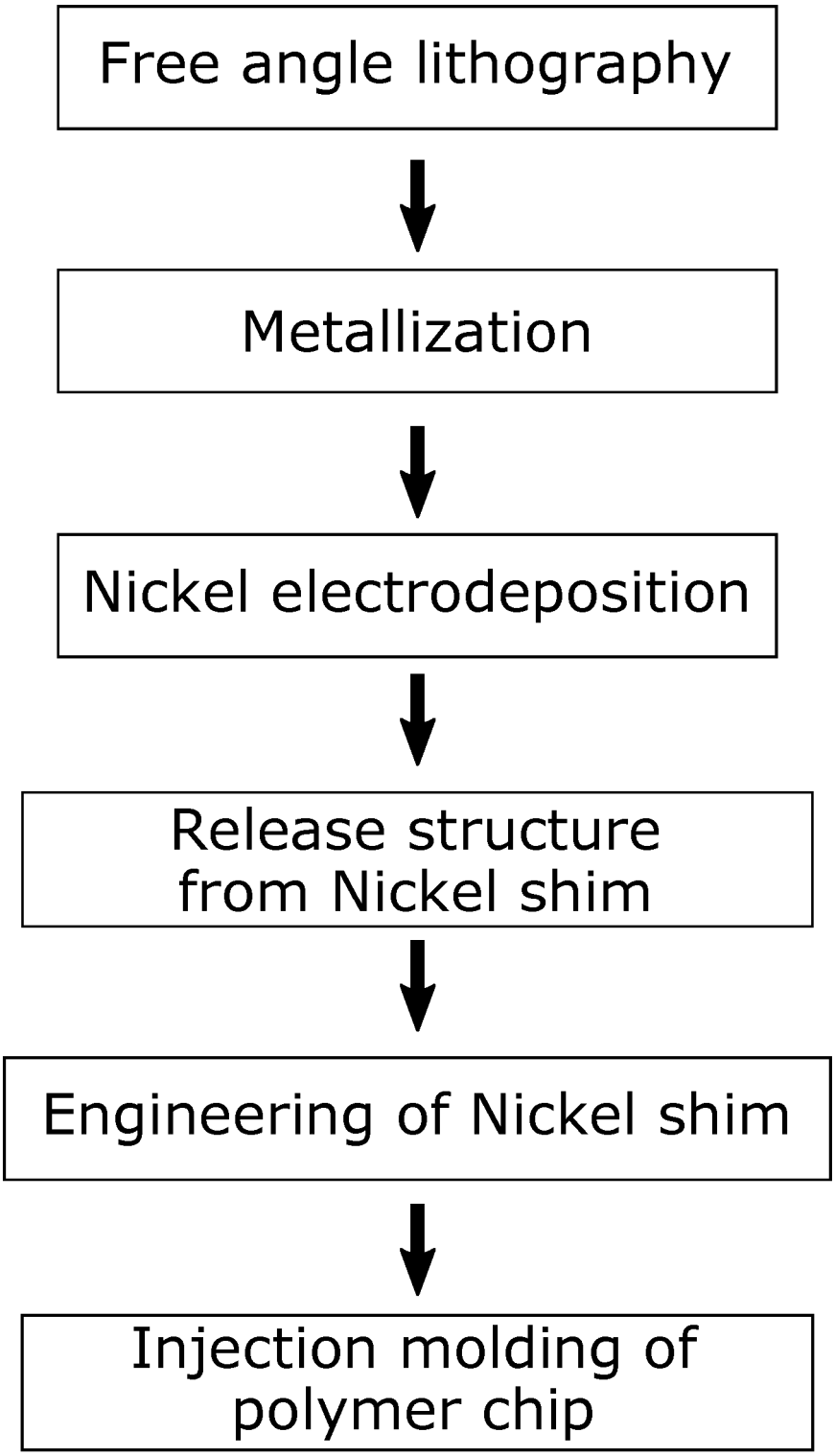

Free angle lithography
Metallization
Nickel electrodeposition
Release structure from Nickel shim
Engineering of Nickel shim
Injection molding of polymer chip

Figure 5

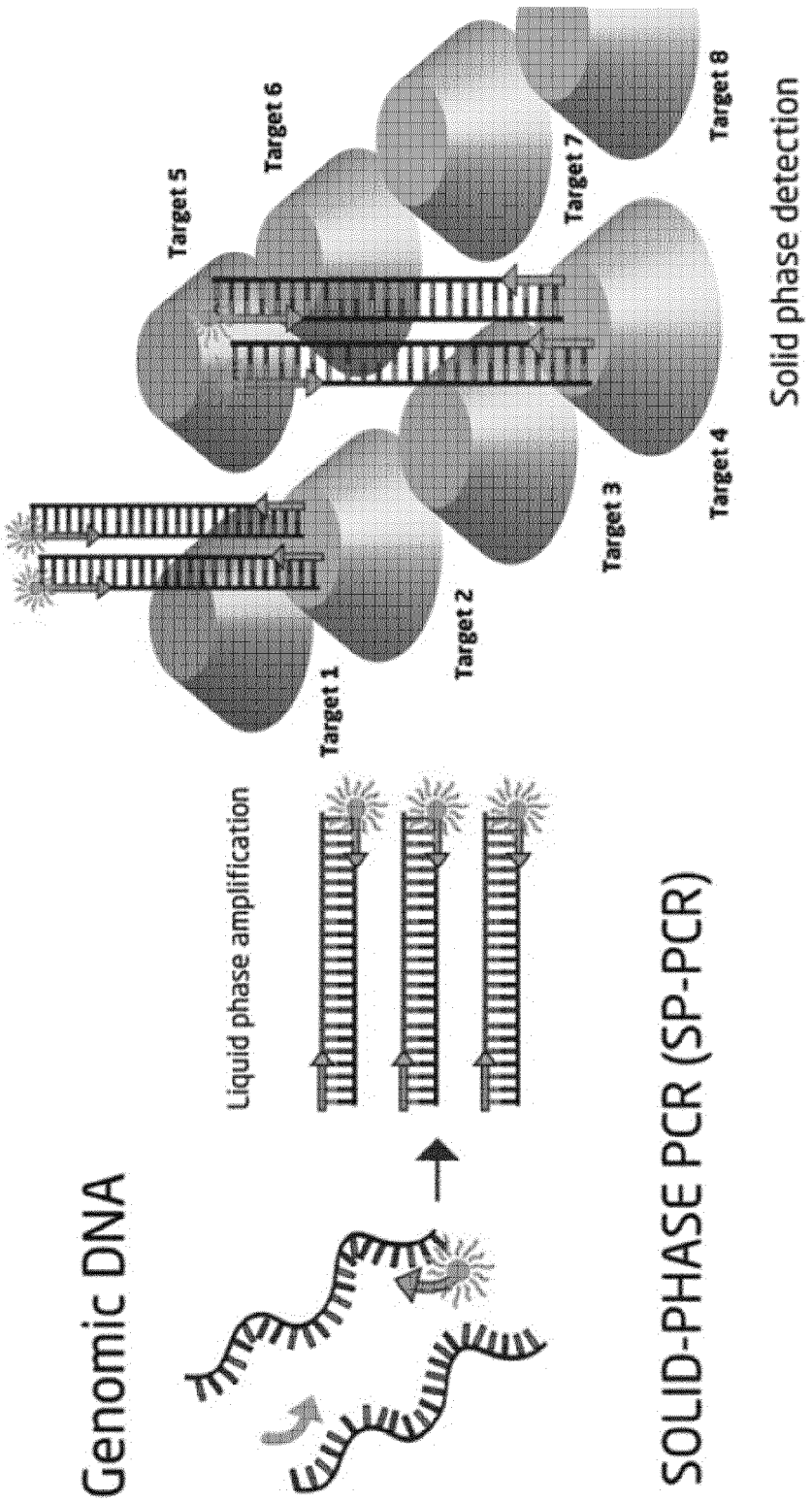

Genomic DNA
Liquid phase amplification
SOLID-PHASE PCR (SP-PCR)
Target 1
Target 2
Target 3
Target 4
Target 5
Target 6
Target 7
Target 8
Solid phase detection

Figure 6

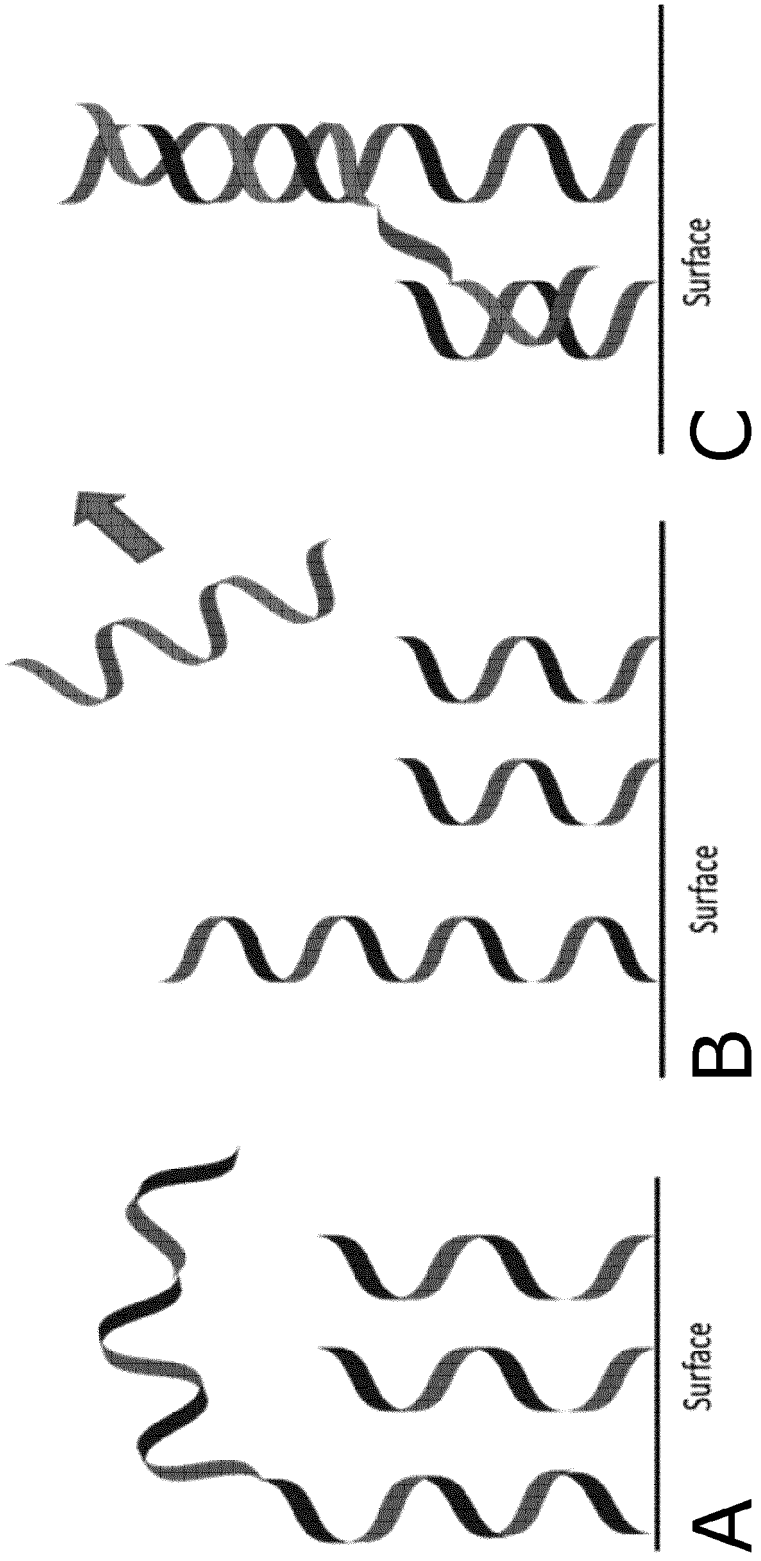


Figure 7

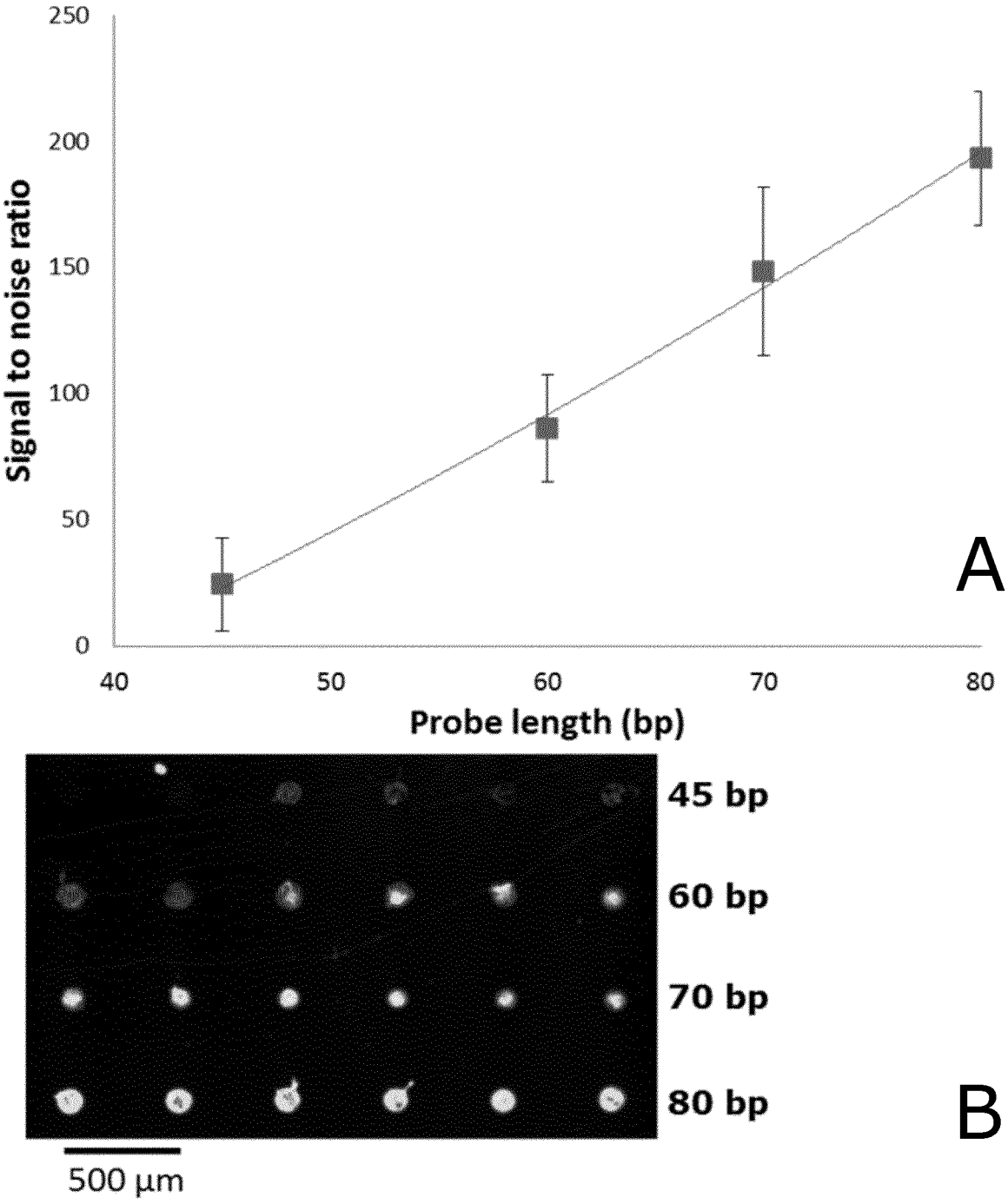

250
200
150
100
50
0
Signal to noise ratio
40
50
60
70
80
Probe length (bp)
A
45 bp
60 bp
70 bp
80 bp
500 µm
B

Figure 8

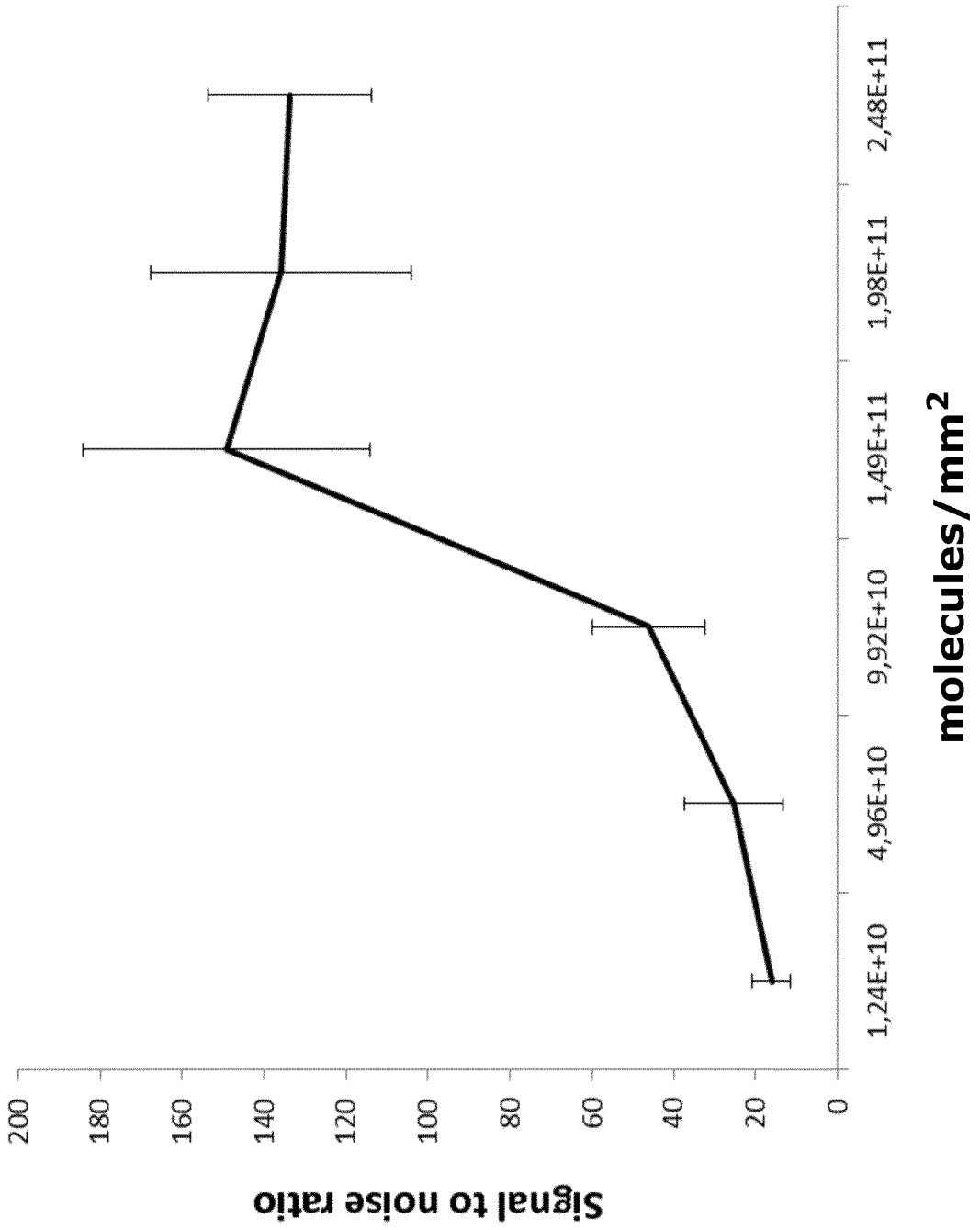

Signal to noise ratio
200
180
160
140
120
100
80
60
40
20
0
1,24E+10
4,96E+10
9,92E+10
1,49E+11
1,98E+11
2,48E+11
molecules/mm2

Figure 9

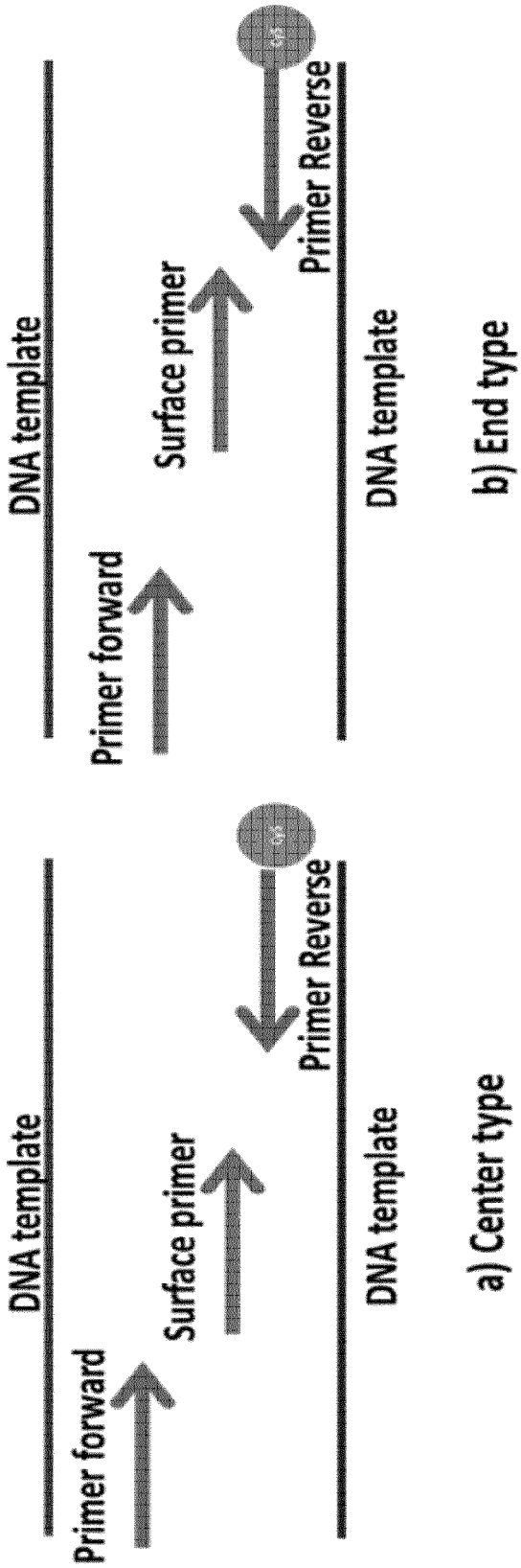

DNA template
Primer forward
Surface primer
Primer Reverse
DNA template
a) Center type
DNA template
Surface primer
Primer Reverse
DNA template
b) End type

Figure 10

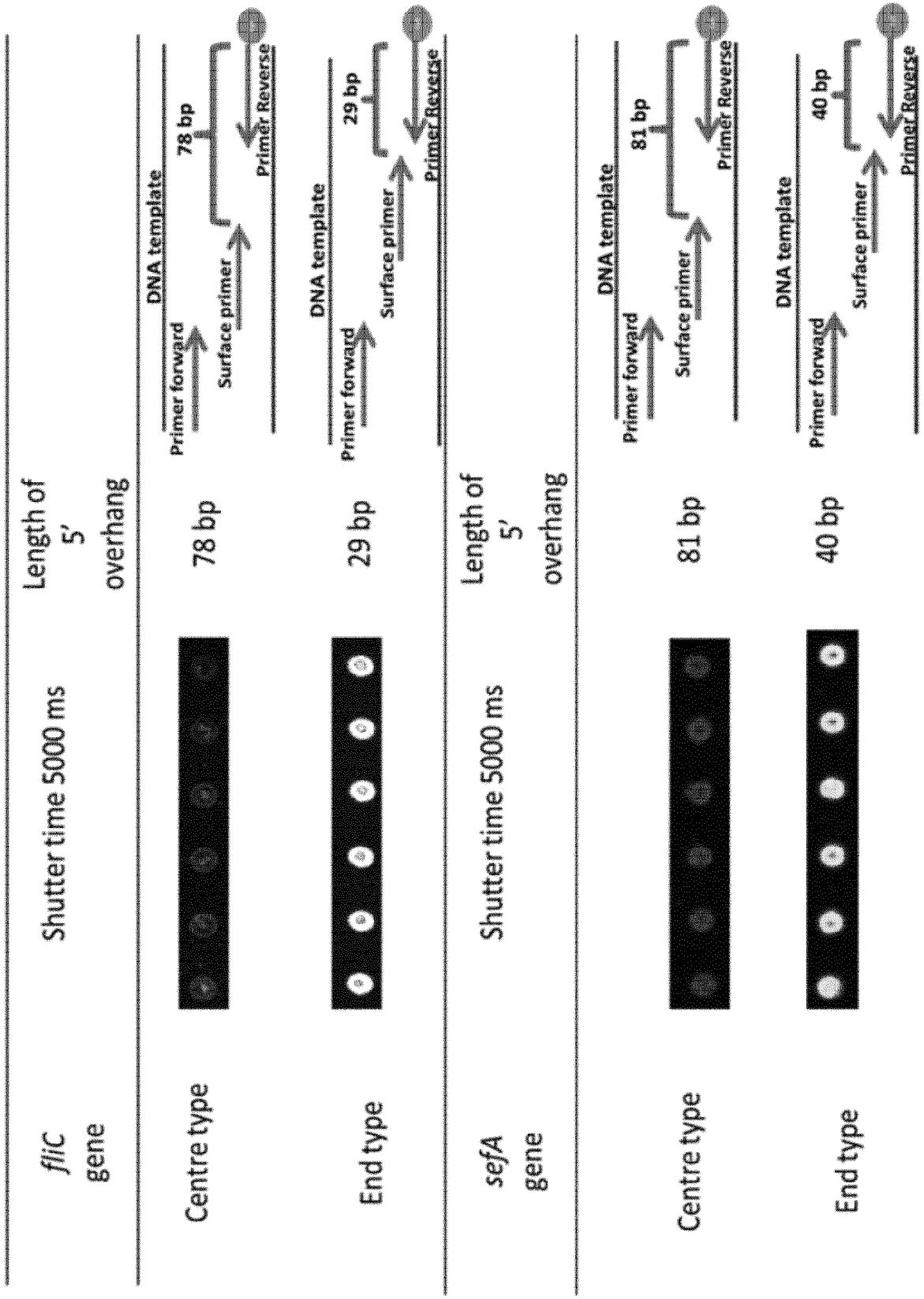

fliC gene
Shutter time 5000 ms
Length of 5' overhang
Centre type
78 bp
End type
29 bp
sefA gene
Shutter time 5000 ms
Length of 5' overhang
Centre type
81 bp
End type
40 bp
DNA template
Primer forward
Surface primer
Primer Reverse
78 bp
29 bp
81 bp
40 bp

Figure 11A

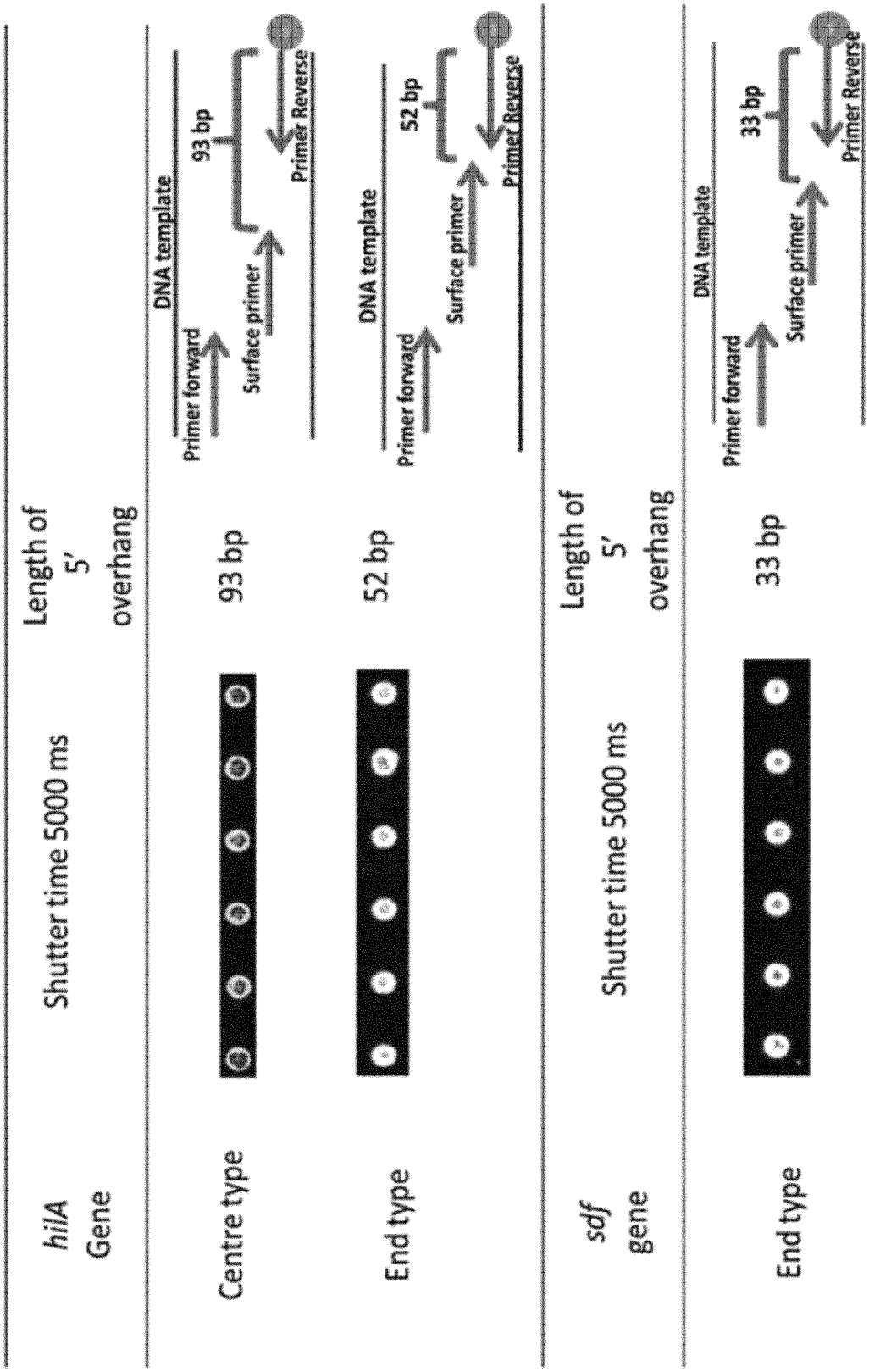
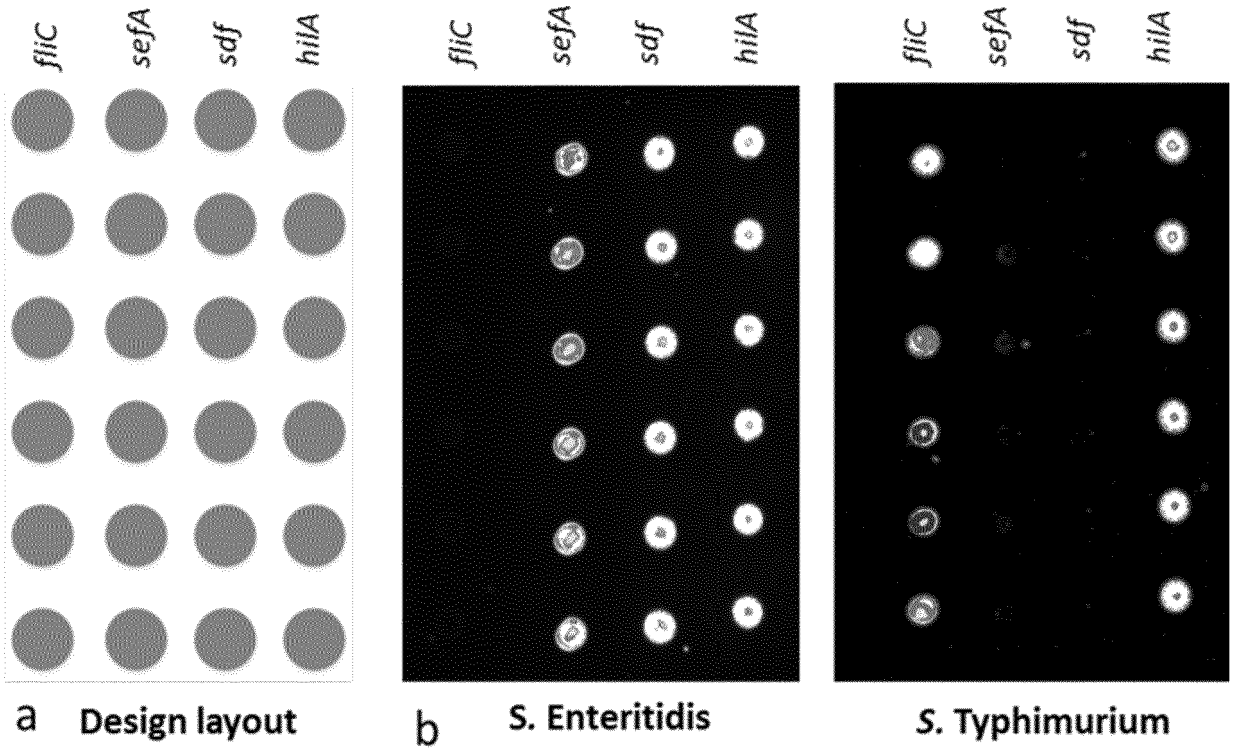
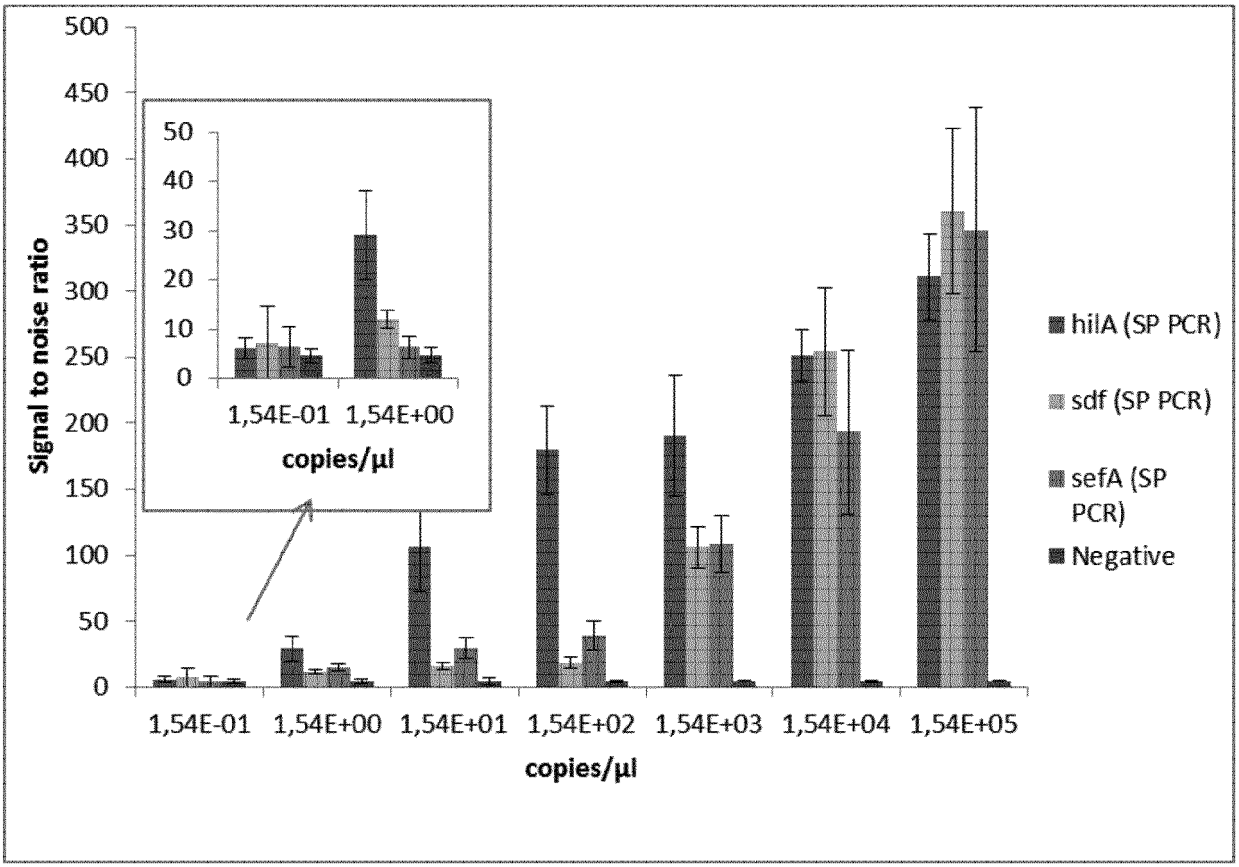

hilA Gene
Shutter time 5000 ms
Length of 5' overhang
Centre type
93 bp
End type
52 bp
DNA template
Primer forward
Surface primer
Primer Reverse
93 bp
52 bp
sdf gene
Shutter time 5000 ms
Length of 5' overhang
End type
33 bp
DNA template
Primer forward
Surface primer
Primer Reverse
33 bp

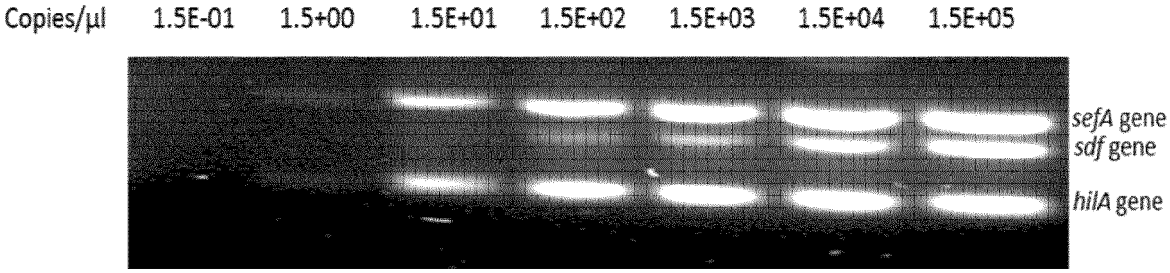
Figure 11B



Species	Primer *fliC* (433bp)	Primer *sefA* (334bp)	Primer *sdf* (299bp)	Primer *hilA* (225bp)
Salmonella spp				X
Salmonella Enteritidis		X	X	X
S. dublin and *S. gallinarum*		X		X
S. Typhimurium and *S. kentucky*	X			X

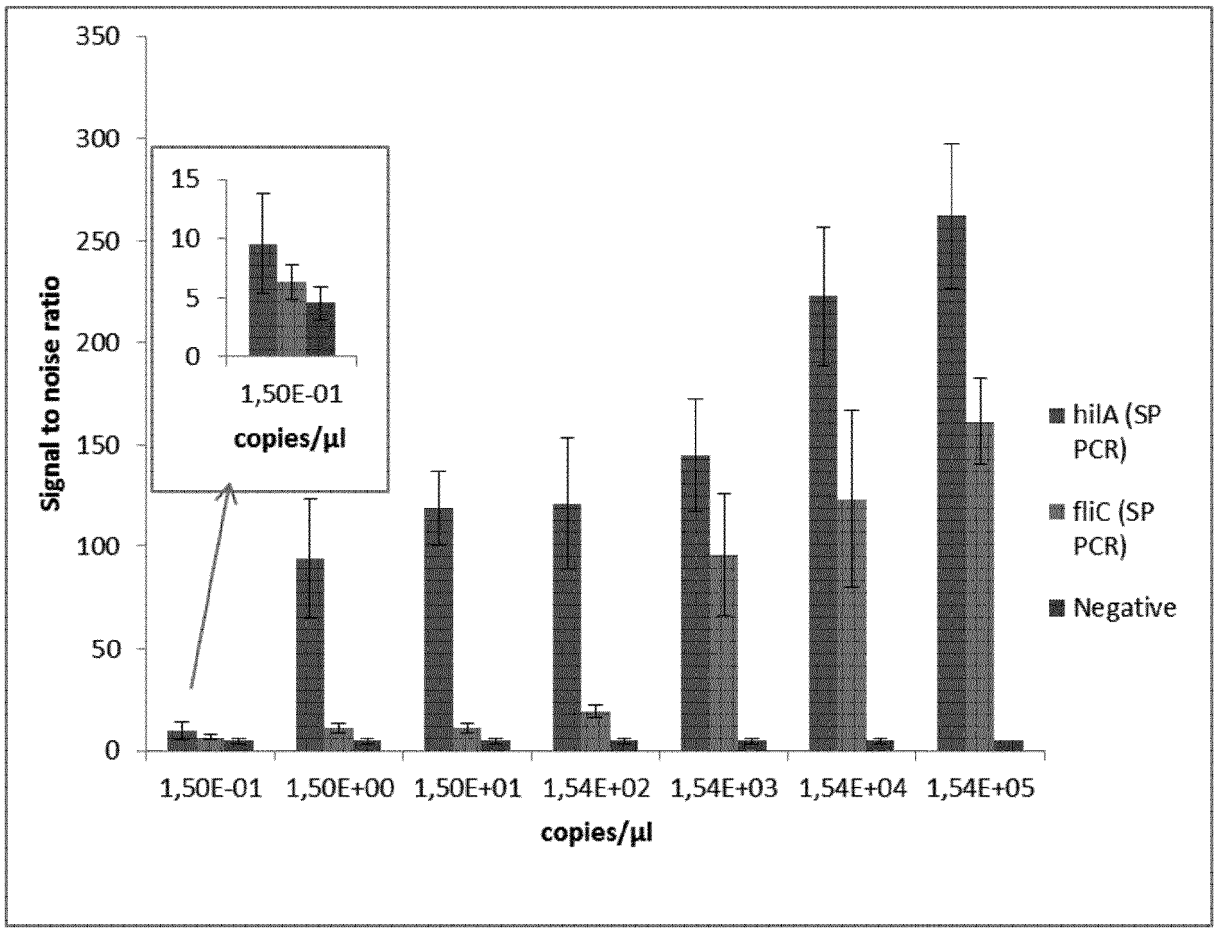
Figure 12

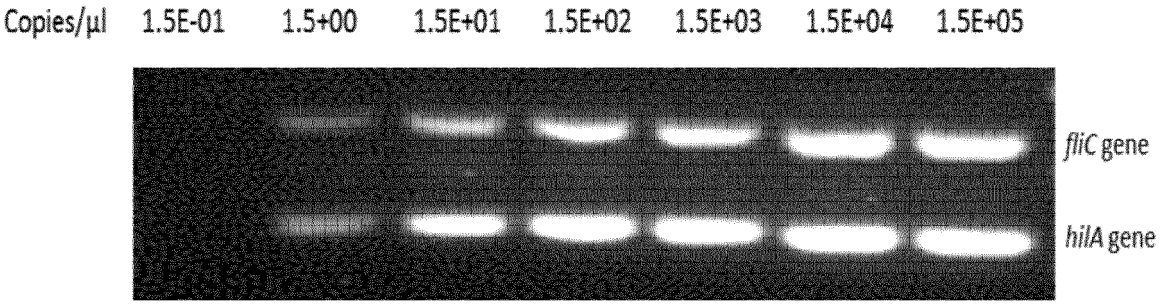




Target	LOD of Liquid PCR (copies/µl)	LOD of SP-PCR (copies/µl)
hilA	1.5E+00	1.5E+00
sdf	1.5E+02	1.5E+00
sefA	1.5E+00	1.5E+00

Figure 13





Target	LOD of Liquid PCR (copies/µl)	LOD of SP-PCR (copies/µl)
hilA	1.5E+00	1.5E+00
fliC	1.5E+00	1.5E+00

Figure 14

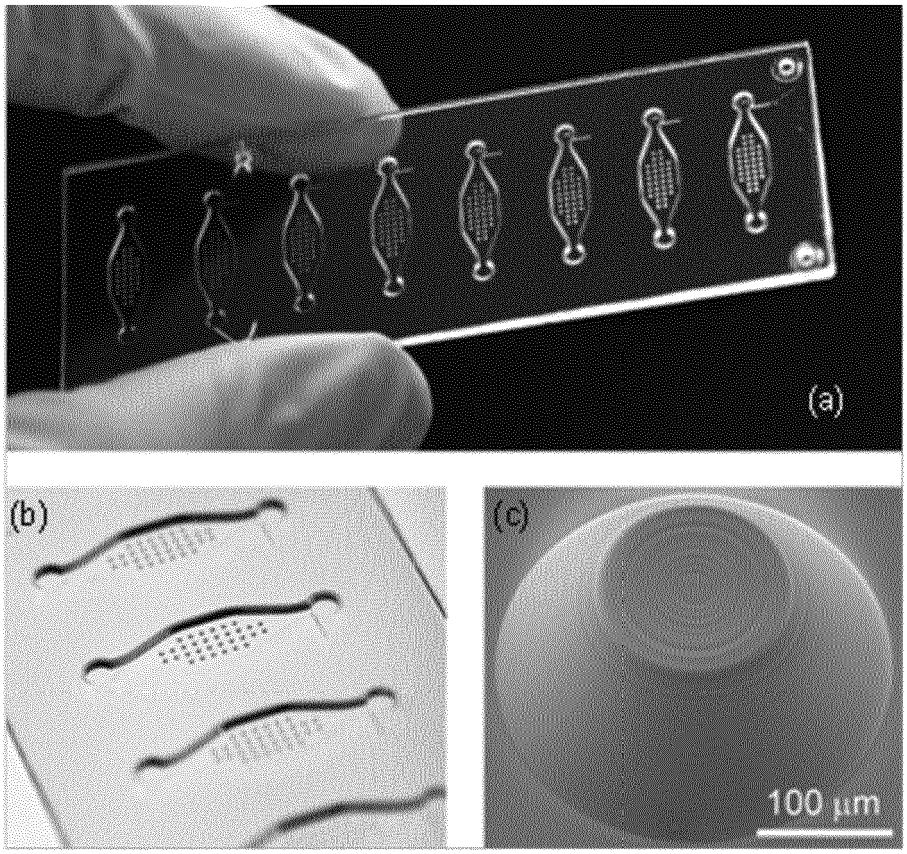
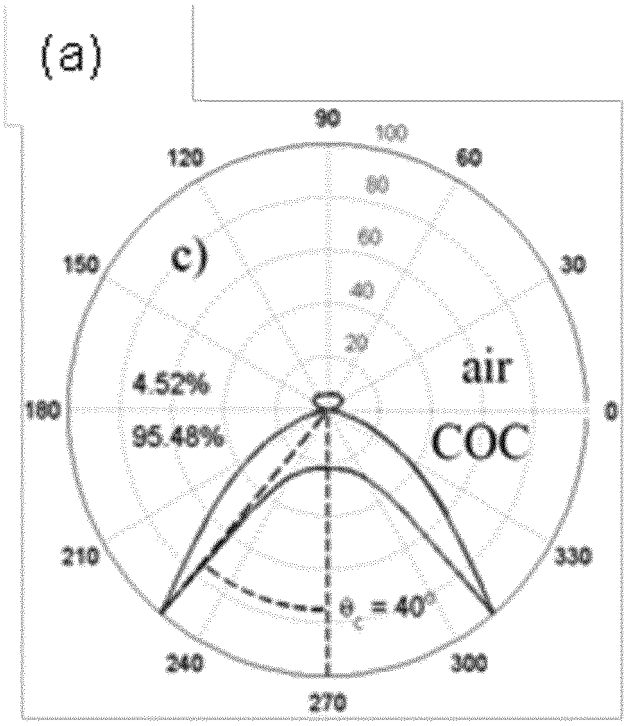


Figure 15



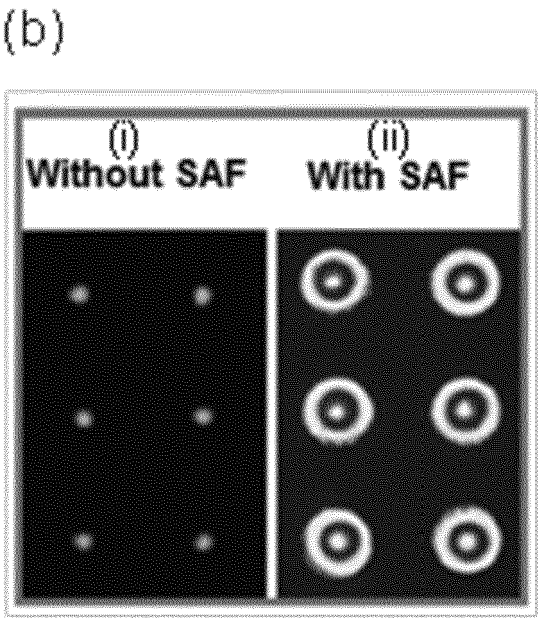


Figure 16A, 16B

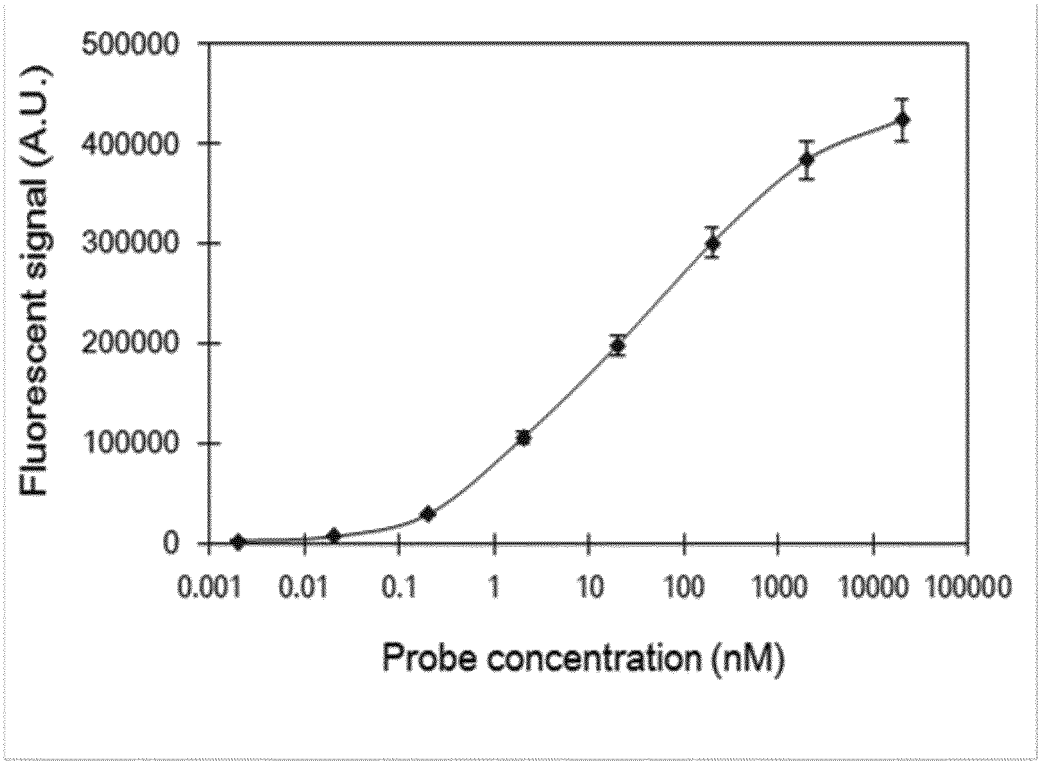
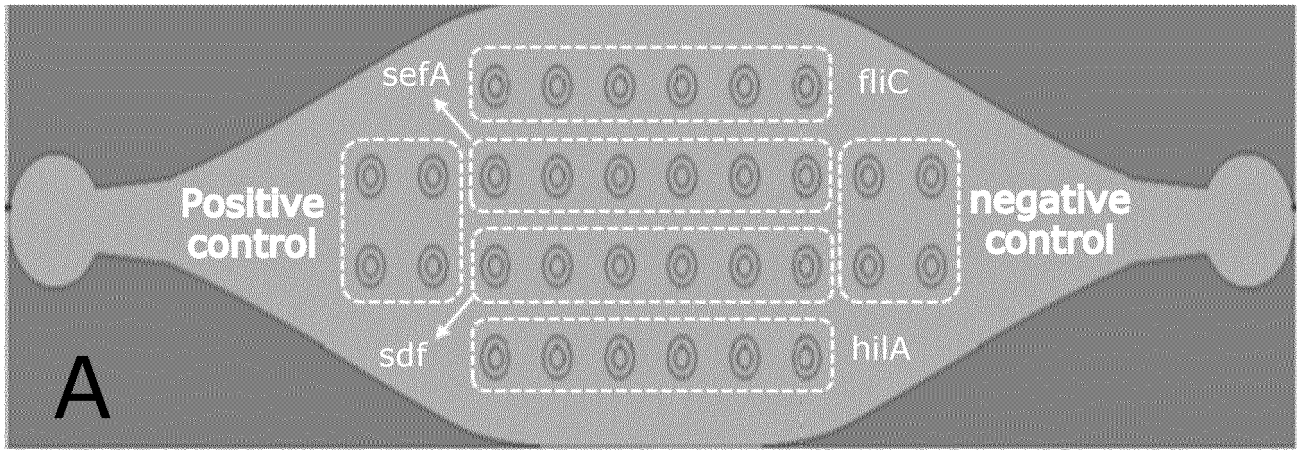
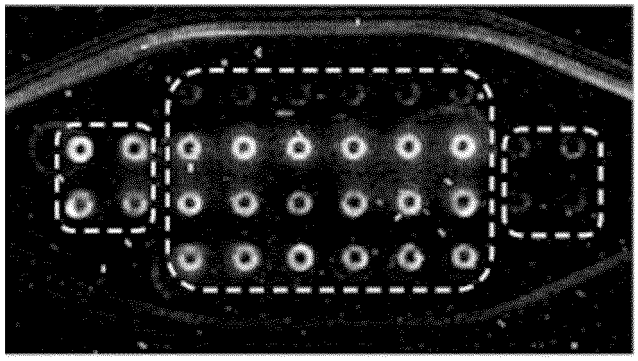
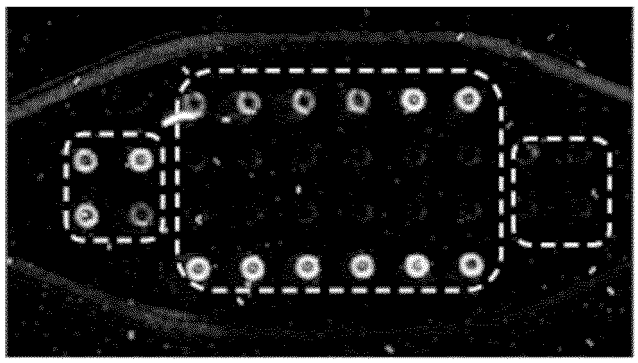

Fluorescent signal (A.U.)
500000
400000
300000
200000
100000
0
0.001
0.01
0.1
1
10
100
1000
10000
100000
Probe concentration (nM)

Figure 16C



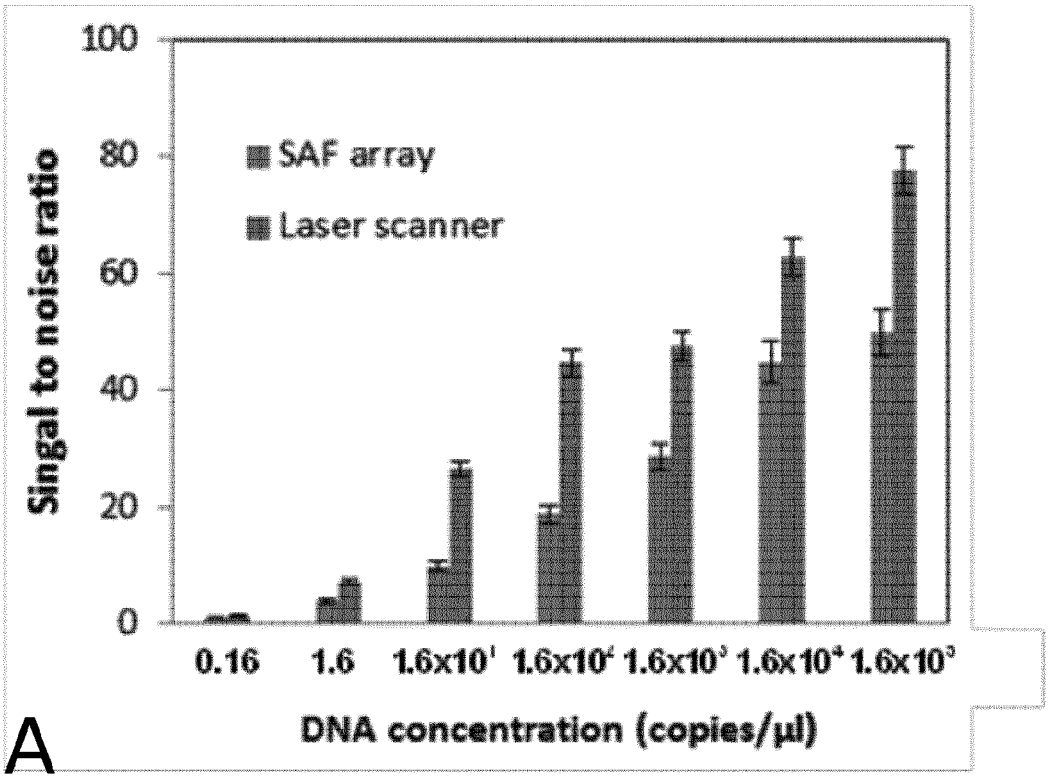


S. Enteritidis



S. Typhimurium

Figure 17



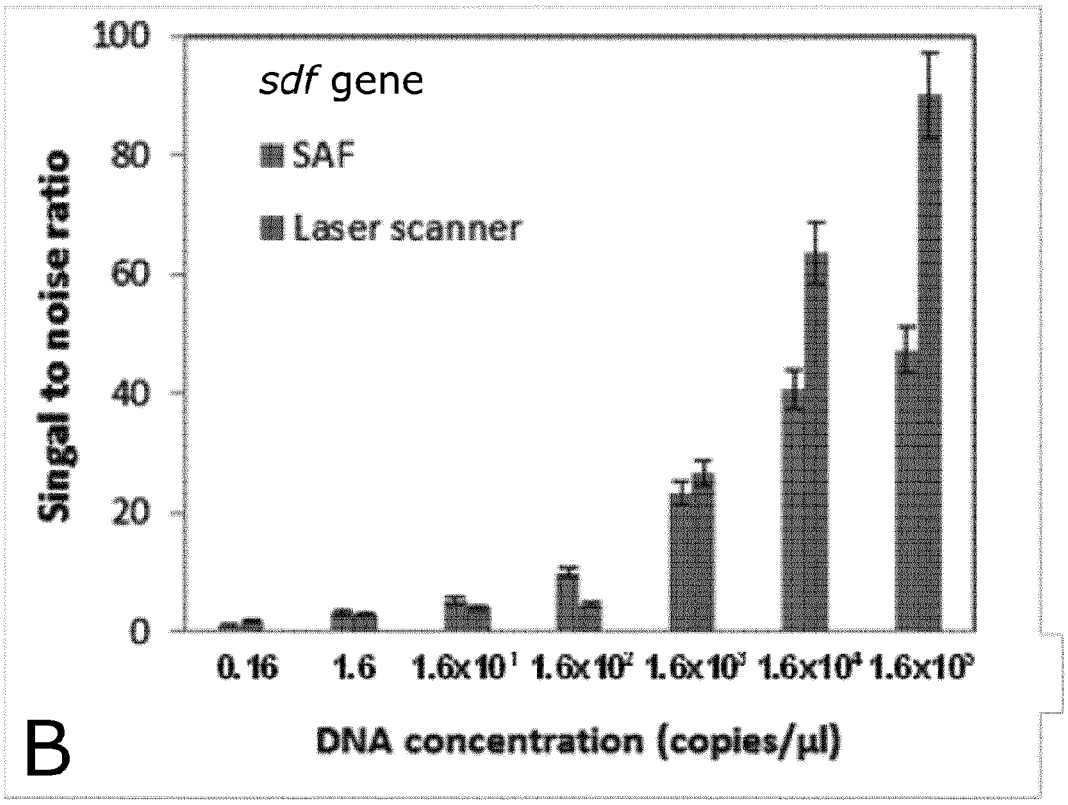


Figure 18A, 18B

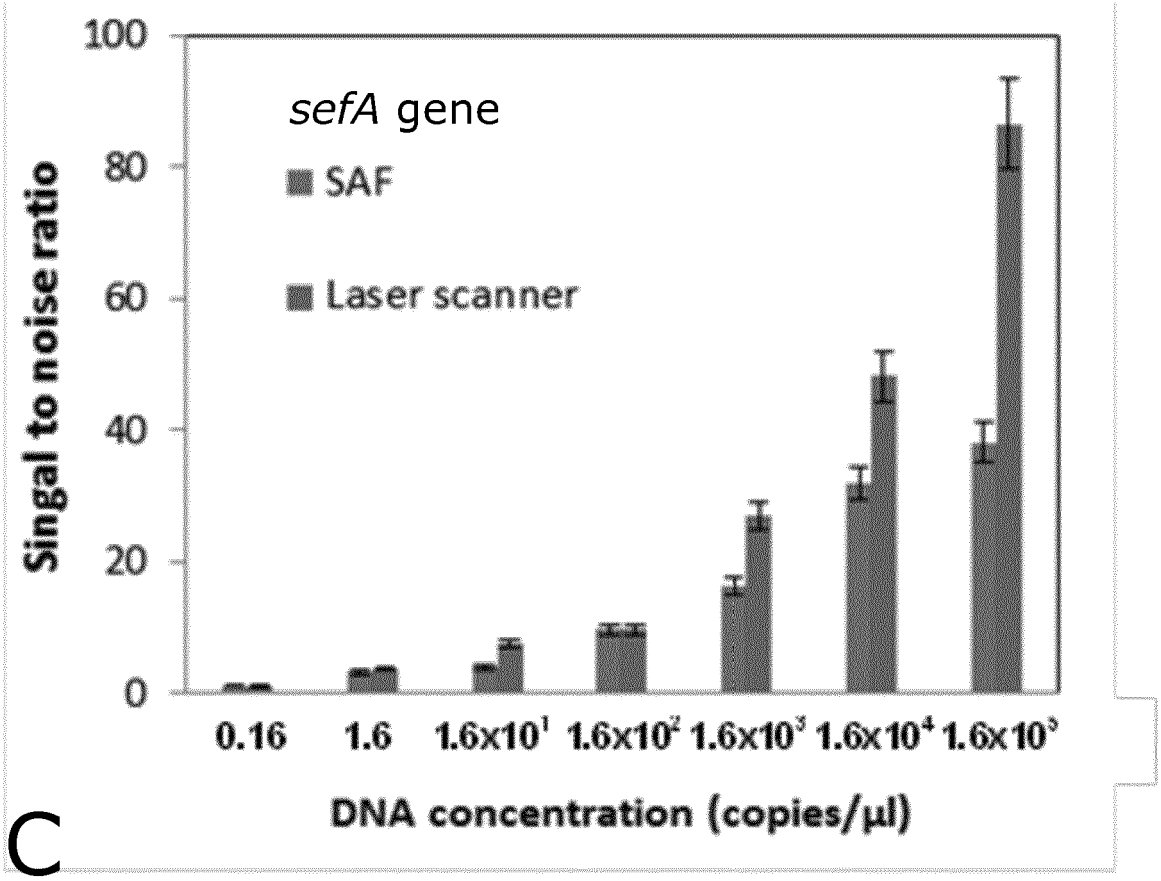

100
80
60
40
20
0
Singal to noise ratio
sefA gene
SAF
Laser scanner
0.16
1.6
1.6x10¹
1.6x10²
1.6x10³
1.6x10⁴
1.6x10⁵
DNA concentration (copies/µl)
C

Figure 18C

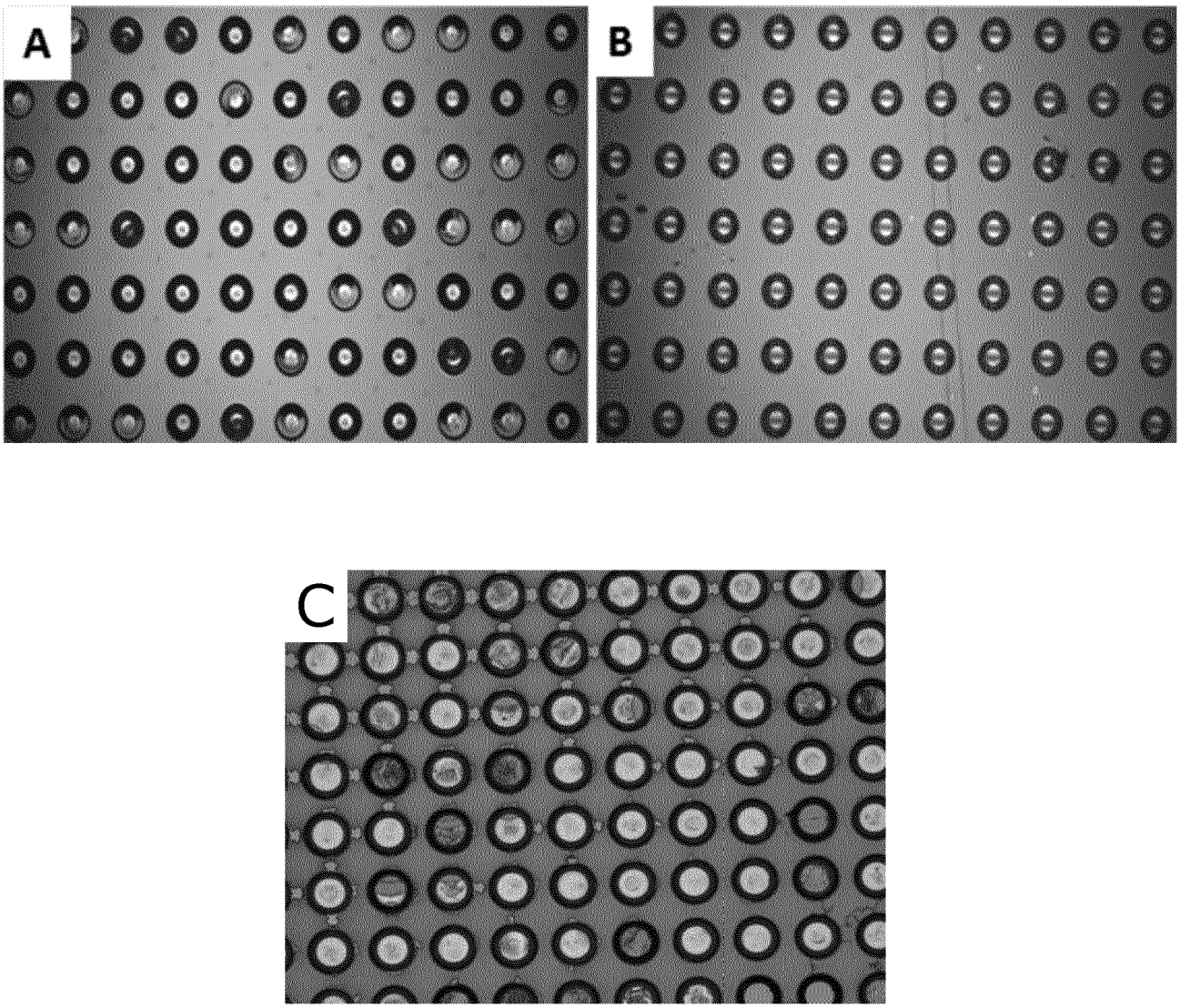


Figure 19

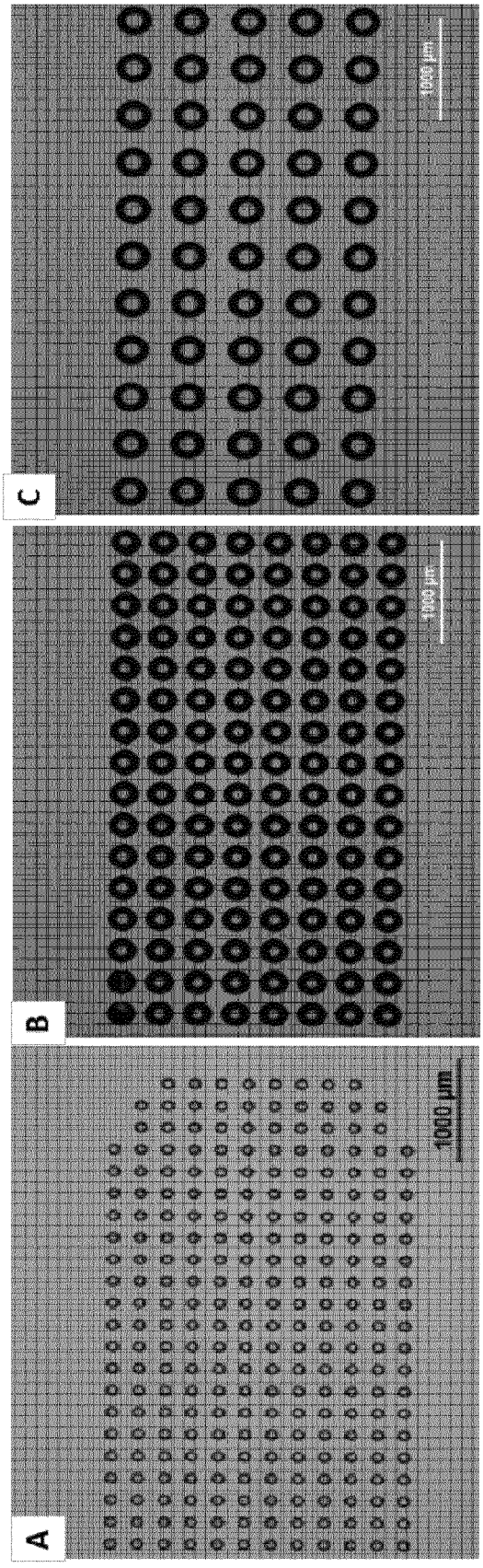


Figure 20

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/072637

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N21/64
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 7 750 316 B2 (MACCRAITH BRIAN [IE] ET AL) 6 July 2010 (2010-07-06) cited in the application abstract; figures 1-3 column 3, line 26 - column 5, line 58	1-16
Y	WO 2009/021964 A2 (UNIV DUBLIN CITY [IE]; MACCRAITH BRIAN [IE]; TRNAVSKY MICHAL [IE]) 19 February 2009 (2009-02-19) abstract; figures 1-8 page 2 - page 3	1-16
Y	GB 2 480 293 A (UNIV DUBLIN CITY [IE]; AMIC AB [SE]) 16 November 2011 (2011-11-16) abstract page 1 - page 5	1-16

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 November 2017	12/12/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pisani, Francesca

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/072637

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JEONG LEE ET AL: "Innovative SU-8 Lithography Techniques and Their Applications", MICROMACHINES, vol. 6, no. 1, 23 December 2014 (2014-12-23), pages 1-18, XP055357525, DOI: 10.3390/mi6010001 abstract paragraph [0001] - paragraph [0002]	1-16
Y	Man Hee Han ET AL: "Fabrication of 3D Microstructures with Single uv Lithography Step", JOURNAL OF SEMICONDUCTOR TECHNOLOGY AND SCIENCE, 1 December 2002 (2002-12-01), pages 268-272, XP055355490, Retrieved from the Internet: URL:http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.511.9990&rep=rep1&type=pdf [retrieved on 2017-03-23] abstract; figure 9 paragraph [0001] - paragraph [0003]	1-16
A	US 2010/243914 A1 (KURZBUCH DIRK [IE] ET AL) 30 September 2010 (2010-09-30) paragraph [0026] - paragraph [0040]	1-16

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/072637

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 7750316 B2	06-07-2010	NONE	
WO 2009021964 A2	19-02-2009	NONE	
GB 2480293 A	16-11-2011	CA 2799159 A1	17-11-2011
		EP 2569613 A2	20-03-2013
		GB 2480293 A	16-11-2011
		JP 5824038 B2	25-11-2015
		JP 2013527451 A	27-06-2013
		US 2013236982 A1	12-09-2013
		WO 2011141530 A2	17-11-2011
US 2010243914 A1	30-09-2010	NONE	